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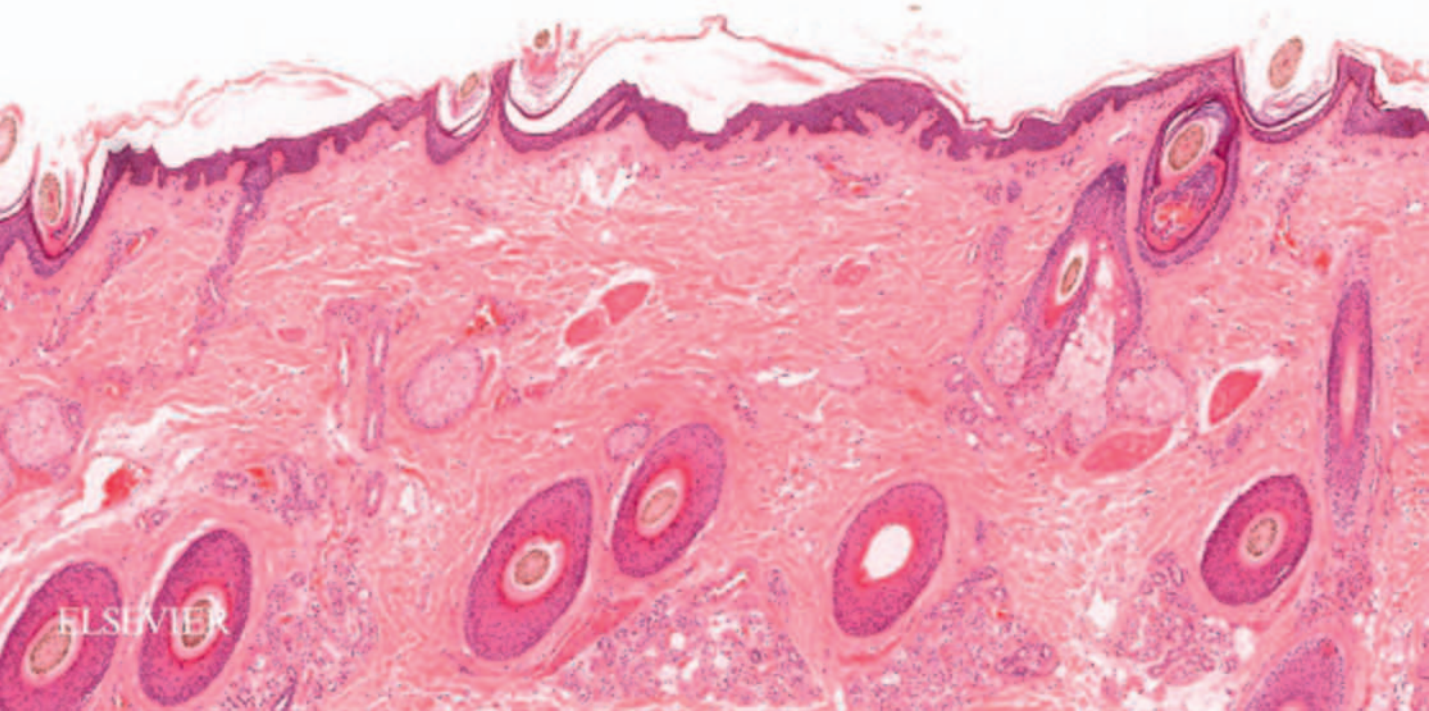
Bancroft's THEORY and PRACTICE of HISTOLOGICAL TECHNIQUES

EIGHTH EDITION



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EIGHTH EDITION

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First edition 1977
Second edition 1982
Third edition 1990
Fourth edition 1996
Fifth edition 2002
Sixth edition 2008
Seventh edition 2013

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ISBN: 978-0-7020-6864-5

Printed in China
Last digit is the print number: 9 8 7 6 5 4 3 2 1

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Preface to the eighth edition

It is now forty years since the first edition of this book was published, and the histological laboratory has changed dramatically in that time. Whilst some techniques of tissue selection, fixation and section production have remained reassuringly constant, there have been great advances in terms of immunological, molecular diagnostic and digital methodology. Immunohistochemistry and immunofluorescence now have well-defined diagnostic and screening roles with quality assurance realities, and are to be found throughout the world with pivotal interactions in patient management. In particular, the progressive development of molecular techniques over the last 20 years, revolving around DNA and in situ hybridization has permitted the creation of new genetic tests and diagnostic opportunities for the laboratory. These are currently at the forefront of guiding treatment choices for patients. At the same time, this has dictated the rational review of some classic histological tests resulting in a reduced histochemical repertoire which is the reality in many laboratories. Digital pathology in particular is the new frontier much as PACS was to radiology 10 years ago. It is likely that the next edition will have a consolidated approach to this exciting new technique.

As always, acknowledgment of the old as well as the new diagnostic methodology will be required by both trained and trainee staff within the histopathology laboratory and scientists in related fields.

As in the 7th edition the classical and now rarely used staining methods are in the appendices but where the reader needs more information, a reference to earlier editions is made. This has allowed for further expansion and update in the newer diagnostic methodologies.

We recognized that some sections on classical stains have not changed dramatically and have simply reviewed these to ensure that their modern relevance has been achieved. The previous edition's three chapters on immunohistochemistry, immunofluorescence and quality control have been amalgamated into one chapter and digital pathology replaces the quantitative data from microscopic specimens. Microarray is now an appendix.

There are several new contributors for this edition. They include updates on the management chapter by Beth Cox and Emma Colgen, and laboratory safety by Ada Feldman. The fixation chapter has been updated by the editors. The immunohistochemistry and immunofluorescent chapter has been updated by the previous authors along with Ann Michelle Cull and Jennifer Marston.

The new chapter on automation is written by Greg Zardin and Lynne Braithwaite, and digital pathology by Jonathan Bury and Jonathan Griffin. Phillipe Taniere, Brendan O'Sullivan, Matthew Evans and Frances Hughes have rewritten and updated the molecular pathology chapter.

Having said this, we are conscious that we are all part of the lineage of authors who have contributed to the previous editions of this book. We salute and thank them for their work. Indeed, their contribution to the success of this ongoing text cannot be underestimated.

Ultimately, we hope that we have produced a modern and relevant histotechnology text which will be of use to those in training as well as established practitioners worldwide. As always, we recognize that this edition is but one step of the ongoing story and hope that our international colleagues will enjoy and approve of the changes which have taken place.

S. Kim Suvarna, Christopher Layton and
John D. Bancroft
March 2018

Preface to the first edition

In recent years histological techniques have become increasingly sophisticated, incorporating a whole variety of specialties, and there has been a corresponding dramatic rise in the level and breadth of knowledge demanded by the examiner of trainees in histology and histopathology technology.

We believe that the time has arrived when no single author can produce a comprehensive book on histology technique sufficiently authoritative in the many differing fields of knowledge with which the technologist must be familiar. Many books exist which are solely devoted to one particular facet such as electron microscopy or autoradiography, and the dedicated technologist will, of course, read these in the process of self-education. Nevertheless the need has arisen for a book which covers the entire spectrum of histology technology, from the principles of tissue fixation and the production of paraffin sections to the more esoteric level of the principles of scanning electron microscopy. It has been our aim then, to produce a book which the trainee technologist can purchase at the beginning of his career and which will remain valuable to him as he rises on the ladder of experience and seniority.

The book has been designed as a comprehensive reference work for those preparing for examinations in histopathology, both in Britain and elsewhere. Although the content is particularly suitable for students working towards the Special Examination in Histopathology of the Institute of Medical Laboratory Sciences, the level is such that more advanced students, along with research workers, histologists, and pathologists, will find the book beneficial. To achieve this we have gathered a team of expert contributors, many of whom have written specialized books or articles on their own subject; most are intimately involved in the teaching of histology and some are examiners in the HNC and Special Examination in Histopathology. The medically qualified contributors are also involved in technician education.

All contributors have taken care to give, where applicable, the theoretical basis of the techniques, for we believe that the standard of their education has risen so remarkably in recent years that the time is surely coming when medical laboratory technicians will be renamed 'medical laboratory scientists'; we hope that the increase in 'scientific' content in parts of this book will assist in this essential transformation.

John D. Bancroft
Alan Stevens
Nottingham, 1977

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The editor(s) would like to acknowledge and offer grateful thanks for the input of all previous editions' contributors, without whom this new edition would not have been possible.

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Acknowledgments

General acknowledgments

Many laboratory scientists and pathologists have contributed in different ways to the seven prior editions of this text and to acknowledge their individual advice and assistance is impossible. We express our thanks to everyone who has contributed since 1977. We owe Harry Cook special thanks for his advice and contributions to the earlier editions. Our thanks are also due to the colleagues we worked with in Nottingham and Sheffield during the production of these books.

We would like to thank all our current authors, and those contributors whose previous work remains in some of the chapters in this new edition. Special thanks go to Richard Horobin who has contributed to all the editions and to Marilyn Gamble for her assistance since the fifth edition. Our thanks also go to those who assisted in the preparation of the manuscripts and the production of the illustrations.

Finally, we wish to thank the staff of our publishers for their unfailing help and courtesy.

John D. Bancroft, S. Kim Suvarna and
Christopher Layton
Nottingham and Sheffield, UK
2018

Acknowledgment to Alan Stevens

I have known Alan since he joined the Pathology Department at the University of Nottingham some 30 years ago; we had many discussions in those early years over whether the time had arrived for a multi-authored text on histological technique. It was apparent at that time that the subject was becoming too diverse for any single or two authors to cover in the depth what was required in the laboratories or the colleges where histotechnologists received their academic education.

In 1977 the first edition of this text was published and was due in no small part to Alan's vision and diligent work in editing and even rewriting some of the chapters. His contributions to the succeeding editions were just as important and his medical knowledge was a significant factor in the development of the book. It has been a great pleasure working with him and I have greatly missed his contribution to the editing of this new edition, although much of his writing in the various chapters remains. The success over the years of Bancroft and Stevens owes a great deal to Alan Stevens. I wish to thank him and wish him well in his current and future medical education publications.

John D. Bancroft
Nottingham, UK
2001

Special acknowledgment

Producing any book involves more than simply the collaboration of a publisher and author/s. There are many people involved in this eighth edition, all contributing to the steady compilation of the text and images that make it a worthwhile reference and state-of-the art commentary.

This special section is to acknowledge one unsung hero of this edition, and indeed most of the previous editions. Carol Bancroft, John's wife, has been a key player throughout this project. Although we have only known her in the last two editions, she has acted as a major force supporting the editors and helping the publication along. She met John whilst doing a BMedSci in the histopathology of lungs of sudden infant deaths, during her medical studies. After qualification, she worked as a General Practitioner.

It is fair to say that we have all relied upon her being able to look over our edits, to use her skills in correcting our grammar, to facilitate streamlining of the many author styles and to identify areas of text which could be better written.

As someone without the daily grind of pathology, she has been ideally placed for these tasks, able to ask non-threatening and incisive questions, free to

direct our efforts and assist us in steadily drawing the book into order. Throughout all these years she has been an unrecognised force and unsung hero, only appreciated by the editorial teams for the individual editions.

For this reason, we have chosen to salute her impressive and unwavering support, and to fully acknowledge her role in the evolution of this project. Without her input, the pathway to this eighth edition would have been harder. We shall always be grateful for her positivity, enthusiasm and forbearance.

S. Kim Suvarna and Christopher Layton
2018

Contents

Pathology laboratory management

1

Beth Cox • Emma Colgan

Introduction

Management of the histopathology laboratory in today's environment requires a balancing act of technical knowledge, business skills, fiscal responsibility, understanding of the workforce and a quality focus. Many of today's managers have 'risen through the ranks' and have a solid foundation of technical skills on which to build, but may need to hone their leadership and management skills. There are excellent books available which cover management issues in depth, see further reading. The objective of this chapter is not to be a comprehensive guide to the subject, but discuss and concentrate on specific areas which are unique and significant to the operation of the laboratory, namely:

- Regulation and Accreditation.
- Quality Management in the laboratory setting.
- Safety concerns specific to Pathology.

The laboratory manager is accountable for the service provided by the laboratory and for the safety and well-being of their staff. It is imperative that the manager remains up-to-date on regulatory and technical changes as well as safety and quality requirements.

Regulation and accreditation

Governmental and national standards have been set to assure that laboratories meet minimal standards in order to protect the public.

In the USA, the Clinical Laboratory Improvement Amendments (CLIA), are federal regulatory standards which apply to all clinical laboratory testing performed on humans. The Centers for Medicare and Medicaid Services (CMS) have responsibility for the operation of the CLIA program with the objective of ensuring quality laboratory testing.

CMS, through CLIA, inspects and certifies all laboratories, either directly or through voluntary organizations with deemed status such as the College of American Pathologists (CAP) or the Joint Commission on Accreditation of Healthcare Organizations (JCAHO). Failure to pass a CLIA, CAP or JCAHO inspection can result in revocation of a laboratory's license to practice. On-site inspections are generally conducted every two years, with self-inspections done in the intermediary years. When deficiencies are discovered, the laboratory is given the opportunity to correct the problem before any disciplinary action is taken. A full catalog of all required standards for the CLIA and CAP can be obtained on line using the websites in further reading. These extensive standards cover all aspects of laboratory operations including the facility, personnel, test performance, safety, quality assurance and quality control. The standards are updated regularly and it is wise to assure that you are working from the most recent set of regulations.

In the UK, the quality of the clinical laboratory service is certificated through accreditation to the laboratory standards pertaining to ISO15189 and previously to Clinical Pathology Accreditation (CPA) standards, rather than through licensing. This is explained further in the section below.

If a laboratory performs any of the licensable activities regulated by the Human Tissue Authority (HTA), such as the processing and storage of post mortem tissue, then an HTA license under the relevant sector is required (for website see further reading). The HTA was established under the Human

Tissue Act and inspects establishments carrying out licensable activities to ensure the requirements of the Act are being met. The establishment must demonstrate at inspection how it meets the standards laid out by the HTA, including quality and governance systems, equipment and facilities in order to retain their licence.

Accreditation

Accreditation in the American pathology laboratory is a voluntary process to confirm that the department meets specific standards for testing, staffing and quality services. Accrediting agencies often have more stringent requirements than the minimal governmental regulations, so laboratories may choose accreditation to identify themselves as a higher quality organization.

CAP is a leading organization which serves patients, pathologists and the public by promoting excellence in the practice of pathology and laboratory medicine worldwide. It provides peer-conducted inspections on a bi-annual basis. Along with accreditation, CAP also offers educational and proficiency testing programs to promote quality practice.

The Joint Commission is an independent, not-for-profit organization which accredits and certifies nearly 21,000 healthcare organizations and programs in the United States. Joint Commission accreditation and certification is recognized as a symbol of quality which reflects an organization's commitment to meet certain performance standards.

The International Organization for Standardization (ISO) standards are being adopted by many as the standards they wish to work to and be accredited by. ISO is the world's largest developer and publisher of international standards which cover many areas of activity; the ones which affect medical laboratories are:

ISO 15189 – *Medical laboratories – Requirements for quality and competence.* This is the main standard which affects medical laboratories and to which the majority will seek to become accredited.

ISO 17043 – *Conformity assessment – General requirements for proficiency testing.* This standard specifies general requirements for the competence of providers of proficiency testing schemes. This includes external quality assurance schemes.

ISO 17011 – *Conformity assessment – General requirements for accreditation bodies accrediting conformity assessment bodies.* To assess and accredit laboratories by ISO standards within their own country. National accreditation bodies such as the CPA in the UK must themselves be accredited under this standard.

ISO 27001 – *Information Security Management Standard (ISMS).* This provides a framework for information security standards. Laboratories may be expected to demonstrate how they conform to these standards when responding to tenders for external work.

In the UK, clinical laboratories are accredited by the United Kingdom Accreditation Service (UKAS) to the ISO15189 standards which cover all aspects of the laboratory, from the management structure of the organization and quality management system to competence of personnel, suitability of equipment and the validity and quality assurance of test methods. Laboratories are assessed on an annual cycle, accreditation is not as a laboratory, but at test level. A list of accredited tests for each clinical laboratory is available on the UKAS website, see further reading.

Unaccredited clinical laboratories may struggle to remain viable if they are unable to demonstrate the quality and accuracy of the test results they provide. The National Health Service (NHS) England advise commissioners to prioritize accredited diagnostic services, stating that accreditation should be seen as the *'baseline standard for diagnostic services across the NHS in England'* (see website in further reading).

Laboratories must also achieve accreditation by the Institute of Biomedical Science (IBMS) if they wish to train persons to become registered Biomedical Scientists. The IBMS inspects laboratories to ensure that they demonstrate the required standard of service and training provision before granting accreditation.

Quality management

A robust quality management system is essential to provide the best possible service for the patient and clinicians. Quality is defined as a measure of how well a product or service does the job for which it is designed, i.e. conformity to specification.

Internal quality control (QC) of work processes is an important part of quality management and has been the traditional way that bench work has been checked for many years. External quality assurance (EQA) schemes provide benchmarking against other laboratories and often provide access to best practice methods and expert advice on improving techniques/specific tests. However, a full quality management system should also encompass systems to ensure consistency, quality of service, confidence, standardization and continual improvement of all laboratory processes. Quality management of a laboratory should ensure that there are systems in place to monitor and improve areas such as organization and quality management systems. This will involve liaison with users, human resources, premises, the local environment, equipment management, information systems and materials. It will address the pre-examination process, the examination process, and the post-examination phase as well as evaluation and quality assurance. Regular audit of the various components of the system will provide evidence of compliance with standards for accreditation. It should identify any trends and issues for concern, and confirm quality systems are working. Taken as a whole, all of these measures should identify areas for quality improvement and show whether any improvements are working.

Quality control (QC)

These systems check that the work process is functioning properly. It includes processes utilized in the laboratory to recognize and eliminate errors ensuring that the quality of work produced by the laboratory conforms to specified requirements prior to its release for diagnosis. Errors and/or deviations from expected results must be documented and include the corrective action taken, if required. In the laboratory, quality control has long been a component of accreditation requirements and should be ingrained in scientists as a daily practice.

Most laboratories have experienced scientists and support staff who have the responsibility of performing routine quality control checks prior to the release of slides for diagnosis. This QC evaluation will include, but is not limited to, accurate patient identification, fixation, adequate processing, appropriate embedding techniques, acceptable microtomy, freedom from artifacts, and inspection of controls to determine the quality and specificity of special staining including immunohistochemistry methods. Criteria should be established which would trigger a repeat if the QC findings were qualitatively or quantitatively unacceptable.

Despite having a conscientious QC system in the laboratory, pathologists perform the final QC examination as they assess/report the slide. It is their responsibility to determine that this is adequate for diagnostic interpretation. However, all personnel are responsible and errors and incidents should be recorded and audited regularly to identify trends. This will highlight any training needs and gaps.

External quality assurance (EQA)

In addition to local data collection and monitoring for internal quality control, external mechanisms provide valuable information regarding quality and peer comparisons and also serve as an educational tool.

In the UK, quality assurance of laboratory techniques is organized on a national basis. It is a system of peer review and registration with appropriate approved schemes. The non-profit-making National External Quality Assurance Scheme (NEQAS) organizes programs for histochemistry and immunohistochemistry. The UK quality assurance schemes were started by members of the profession in order to establish quality standards within histopathology. Registration with the schemes is now a requirement for accreditation. The quality assurance process is based on peer review of the stained sections submitted by participating laboratories. There are also medical quality assurance schemes for pathologists which cover many of the sub-specialties of histopathology. The quality assurance schemes currently

used in the UK are coordinated under the auspices of UK NEQAS. Within this organization there are two individual schemes for histopathology, NEQAS for immunohistochemistry and NEQAS for cellular pathology techniques. The immunohistochemistry scheme gives participants the option to be assessed on general antibody panels or more specialist laboratories may choose to participate only in their specific areas. The cellular pathology scheme is subdivided into general, veterinary and neuropathology.

In the USA, the National Society of Histotechnology (NSH) in partnership with the College of American Pathologists (CAP) created the Histology Quality Improvement Program (HistoQIP). This quality assurance system scores each slide, assessing the fixation, processing, embedding, microtomy, staining and coverslipping for routine H&E slides, special stains and immunohistochemistry. Participants receive an evaluation specific to their laboratory, an educational critique and a participant summary report which includes peer comparison data, performance benchmarking data and information regarding best-performing procedures and techniques. Additionally, CAP establishes national surveys for immunohistochemistry.

Accreditation standards require that action is taken by poor performers to improve the quality of their preparations. Most schemes offer expert assistance and advice to laboratories which fall below the defined acceptable score.

Process improvement

This is the system which is used proactively to approach and identify opportunities to improve quality before problems occur. It operates through evaluation and audit of all systems and processes in the laboratory. The goal is to improve care and safety for patients and staff through recognition of potential problems and errors before they can occur. Good managers realize that failures, errors and problems are often due to the system processes, and not necessarily the fault of the employee(s).

Regular and thorough auditing of the many components of the laboratory's quality management system and performance should be mapped against accreditation standards. This will help highlight any problem areas. Feedback from users provides useful information when evaluating the effectiveness and quality of the service. Any criticism received may prompt an unscheduled audit of that part of the system.

Continuous Quality Improvement (CQI) should include auditing of the laboratory's procedures against, not only accreditation standards, but also those of the host organization/other services. Any audit findings which show that the laboratory's processes are not adequate should result in corrective actions. These audit findings may also highlight opportunities for improvements in processes, documentation, staff training, or monitoring aspects of competence. Any corrective actions required should be completed as soon as possible so that services can be improved and brought up to the correct standard quickly. CQI is a continuous cycle of audit and assessment of the service. If not monitored regularly, quality standards can slip as staff, equipment and reagents change. It is useful for the manager to establish an audit calendar to ensure that all areas are audited regularly, paying particular attention to 'problem areas'.

Risk management

Risk management is an essential and central part of all laboratory work. To comply with legislation and maintain accreditation a laboratory must have an effective risk management policy. Any chance of something going wrong should be either negated or minimized and therefore a laboratory's risk management process should have procedures in place for:

- Identifying all risks which exist within the environment.
- Assessing those risks for likelihood and severity.
- Eliminating those risks which can be removed.
- Reducing the effect of risks which cannot be eliminated.

The pathology laboratory should have close links with, and feed into, the host organization's risk management process. In most hospital laboratories,

the laboratory manager will be accountable for risk management and may be supported by a Risk Lead who will be responsible for the operational aspects of the system. To function effectively and safely, all the laboratory procedures and activities must be subjected to the risk management process. The risks in the laboratory are similar worldwide, albeit with a variation due to local circumstances. Health and Safety, as well as quality assurance, incorporate a major aspect of risk management. All aspects of our working life incorporate a degree of risk, and the risk management process allows us to prioritize, evaluate and handle the risk appropriately. It is not possible to avoid or eliminate all risks and in reality, this may not be practical. It is important to identify and understand the risks which are involved in a laboratory's working practices.

A laboratory manager should be concerned with all the risks associated with the department they manage, and also how these may impact on other areas of the organization such as porters transporting samples or chemicals to the laboratory. The laboratory manager would also be required to alert the organization to the presence of risks which cannot be adequately controlled within or by the department.

The laboratory management team will deal with any laboratory associated risk by ensuring that adequate resources are available to deliver a service which is safe for both staff and patients. Staffing levels and competence, timeliness and quality of results, budgetary management, consumable and equipment supplies, and maintenance are some of the areas of concern. The laboratory management team must also ensure that risk management procedures are in place for every aspect of a laboratory's processes and its environment.

The laboratory manager must ensure that day-to-day errors do not arise because of inadequacies in laboratory procedures and that quality control checks are in place to minimize the possibility of human errors, e.g. a transcription error or mislabeling. Standard operating procedures (SOPs) should include Control of Substances Hazardous to Health (COSHH) data, risk assessments or equivalent, and also other health and safety information relevant to the procedure. This should include national legislation and guidance where available. It is important that where risks are identified, the risk management measures are regularly audited to assess whether they are being followed and that they remain appropriate and effective.

Risk identification

The risks within each laboratory section are best identified by the section lead and members of that team working in conjunction with the laboratory's Health and Safety lead. This ensures that the broadest possible spectrum of viewpoints is considered. During this process, it is useful to divide the risks into different categories. These include clinical, physical, chemical, infectious, and even organizational, financial and political, depending on the area being risk assessed. For example, a support worker unpacking the samples delivered to the laboratory may have noticed that samples have leaked, possibly putting both themselves and the porter at risk from infection and exposure to the fixative; if any of the contents have leaked beyond the specimen bag, there could be a risk to other health workers and patients/visitors using the same route. This could just be a problem with one batch of specimen containers, but could also be a training issue for staff putting the samples into the containers. In raising the issue with their supervisor and giving them the opportunity to investigate the root cause, the support worker may have prevented harm to others and potential damage to the sample, which may impede its diagnosis.

Risk analysis and evaluation

Analysis and evaluation of potential risks is an essential part of the process and one that is used to identify both the likelihood and severity of these risks. By scoring the risks for likelihood and severity, it is then possible to use a matrix such as the one described below as a tool which will put a value on specific risks. This will then help prioritize them for further action.

The risk manager should put a system in place whereby all incidents and accidents are reported no matter how small. It is only by recording all the data that the full picture can be obtained and analyzed

and areas possibly overlooked initially, be risk assessed and managed.

There are numerous ways of controlling risk, but frequently there will be expert guidance or regulations issued by professional bodies or government agencies which the Risk Lead should ensure are implemented. Informal networking with professionals in similar laboratories can also provide valuable information and ideas as to how others have overcome the challenges of managing risks (see Risk assessment tool below).

Risk Assessment Tool

Severity and likelihood values

The following is an example of a severity scoring scale for incidents:

1. Low

- Minor injury or harm.
- Minor loss of non-critical service.
- Minor non-compliance with standards.
- Minor out-of-court settlement.
- Publicity mostly contained within organization. Local press coverage of no more than one day.

2. Slight

- Injury or harm requiring less than 3 days absence from work, or less than 2 days hospital stay.
- Loss of service for less than 2 hours in several non-critical areas, or less than 6 hours in one area.
- Single failure to meet internal standards.
- Civil action with or without defense, improvement notice.
- Regulatory concern.
- Local media coverage of 2 to 7 days.

3. Moderate

- Medical treatment required and more than 3 days absence from work, or more than 2 days extended hospital stay.
- Loss of services in any critical area.
- Repeated failures to meet internal standards or follow protocols.
- Class action, criminal prosecution or prohibition notice served.

4. Severe

- Fatality, permanent disability or multiple injuries.
- Extended loss of essential service in more than one critical area.
- Failure to meet national standards.
- Executive officer fined or imprisoned, criminal prosecution and no defense.
- Political concern, questions in parliament, national media coverage longer than 3 days.

5. Catastrophic

- Multiple fatalities.
- Loss of multiple essential services in critical areas.
- Failure to meet professional standards.
- Imprisonment of executive from organization.
- Full public enquiry.

Incidents may also be scored 1–5 for likelihood:

1. Incident unlikely to occur.
2. Incident likely to occur once in a 5 year period.
3. Incident likely to occur yearly.
4. Incident likely to occur once in a 6 month period.
5. Incident likely to occur once every 4 weeks or more frequently.

The Risk Factor is the severity, multiplied by the likelihood of occurrence:

Very Low Risk, 1–2 – The majority of control measures in place, or harm or severity small. Action may be long term.

Low Risk, 3–4 – Moderate probability of major harm or high probability of minor harm if control measures are not implemented. Action in the medium term.

Moderate Risk, 5–10 – Urgent action to remove or reduce the risk.

High Risk, 12–25 – Immediate action to remove/reduce the risk.

Audit

This is an essential tool in risk management. Regular audits of the effectiveness of the risk management measures put in place, and the frequency and nature of incidents, will allow the laboratory's risk management team to assess them, and amend and improve if required. Audit will also identify areas or tasks which may need additional or increased monitoring, and may highlight training gaps for individuals or groups of staff. In addition, regular and targeted audits will provide evidence to assist with driving change should the risk be due to the lack of funding

for certain tasks or processes, or to identifying processes outside the control of the laboratory management, e.g. labeling of samples in the operating room.

Risk funding

Risk management should also consider insurance, individual or laboratory, as an important option. All medical staff carry medical liability insurance which covers them in the event of any negligence claims. Similarly, professional indemnity insurance is commonly available today for non-medical laboratory staff who are much more at risk in today's litigation conscious society. The decision regarding whether or not to insure should be based on the risk assessment and the severity and likelihood of the risk. Some risks will not be appropriate for insurance coverage and in these instances the risk must be accepted by the organization.

Safety

The Pathology laboratory manager is responsible for the safety and well-being of all the staff and visitors to the department. Safety concerns include the common, e.g. slips and falls, and those more specific to histopathology. Pathology practice commonly includes a multitude of physical, biologic and chemical hazards which must be evaluated and eliminated or reduced. Elimination is possible, e.g. by looking for alternatives to high-risk, harmful chemicals used in the laboratory.

Where hazards remain, efforts should be made to reduce the effect or possibility of the risk. Consideration must be given to altering work practices, making engineering changes and use of Personal Protective Equipment (PPE). There are numerous ways of controlling hazards and frequently there is expert guidance or regulations issued by professional bodies or government agencies which the Safety Officer should ensure are implemented. Informal networking with professionals in similar laboratories can also provide valuable information and ideas as to how others have overcome the challenges of managing certain risks.

In the UK, organizations such as the Health and Safety Executive (HSE) and the Health Protection Agency (HPA) exist to ensure the safety of employees, patients and the general public. Regulations made under the Health and Safety at Work Act 1974 apply to all work situations, e.g. COSHH regulations and the Workplace (Health, Safety and Welfare) Regulations. The HSE enforces this act along with others, including the Health and Safety Offences Act 2008.

The overall message is that employees are entitled to work in environments where risks to their health and safety are properly controlled, i.e. minimized. Under health and safety law, the primary responsibility is owed by employers, with employees expected to ensure their own safety and that of their colleagues and/or patient's by adhering to policies and procedures.

In the USA, the mission of the Occupational Safety and Health Administration (OSHA) is to prevent work-related injuries, illnesses and occupational fatality by issuing and enforcing standards for workplace safety and health. Most countries will have equivalent bodies and standards. OSHA guidelines require:

- Availability and maintenance of safety equipment.
- Measures to control the risk of exposure to chemical hazards or biological specimens by employees of the laboratory testing facility.
- Sanitary condition of the testing laboratory.
- Annual inspections.
- Waste management program.
- Procedures for infectious material response, ventilation failure, first aid, fires or emergencies.
- Documentation of all spills and exposure incidents.

To comply with legislation and maintain accreditation, a laboratory must have an effective safety program or risk management policy. Any chance of something going wrong should be either negated or minimized and therefore, a laboratory's safety management process should have procedures in place for:

- Identifying all risks which exist within the environment.
- Monitoring and assessing those risks for likelihood and severity.
- Eliminating those risks which can be removed.
- Reducing the effect of risks which cannot be eliminated.

The pathology laboratory should have close links with, and feed into, the host organization's safety

program or risk management process. In most hospital laboratories, the laboratory manager will be accountable for the health and safety of the staff in their department and often will be supported by a Safety Officer who will be responsible for the operational aspects of the system.

In the USA, the 'Right to Know' law is the legal principle giving an individual the right to know the chemicals to which they may be exposed in their daily living and work life. This law requires that employees be trained on the hazards they work with and know which precautions should be used to prevent exposure.

Safety Data Sheets are required to be supplied by all chemical manufacturers and they must be available for all the employees in the laboratory. These will provide comprehensive information about a substance or mixture for use in workplace chemical management.

Xylene and formalin exposures in pathology are one of the common concerns. Along with reducing exposure by altering work practices, engineering changes and the use of personal protective equipment, vapor monitoring must be done on a regular basis, usually annually, or when there has been a change within the laboratory. This is often done by employees wearing a monitoring badge during their work tasks which is then evaluated by an independent laboratory. Results may be expressed as an 8-hour Time Weighted Average (TWA) or as Short Term Evaluation Limit (STEL). See also Chapter 2, Chemical Safety in the Laboratory.

Laboratory procedures

In most laboratories, SOPs must be carefully established to comply with regulatory standards. A laboratory's testing procedures may be multiple and complex and it is essential that the methodology for all the procedures and tests are documented in SOPs allowing all staff to operate in a standardized and appropriate way. SOPs should cover all aspects of the testing process from delivery and storage of samples or reagents, to the issuing of the final laboratory report. The SOPs include not only the laboratory procedures but also those carried out by the pathologists and clerical staff. It is important that SOPs which impact on areas of staff outside of the laboratory, e.g. porters delivering samples from operating rooms, are shared with the other departments responsible for managing that part of the process.

Accreditation standards require that SOPs and other policies are within a document control system. This is usually a central database which holds authorized copies of documents, with controls on who can modify these data. The document control system must also ensure that only authorized and up-to-date copies of SOPs and policies are being used by staff performing the tasks. Any changes to a procedure must be recorded within a further updated SOP. This must then be issued and any old SOPs removed from circulation.

Departmental organization

An appropriate management structure for the department should exist ensuring that the main functions can be adequately delivered. Staff at all levels should be qualified and trained for the work they do, and hold appropriate registration, if required. Competencies for the tasks performed should be regularly assessed, checked and recorded. Many departments publish a mission statement outlining their business and aims. Quality objectives need to be documented so that all have clear objectives outlining who is responsible for achieving them and when they should be achieved by.

A laboratory will have multiple users, including patients, clinicians and those purchasing its services. It is essential when planning and developing a laboratory service that all users are consulted. Likewise, when monitoring the effectiveness and quality of a service, user feedback should be sought so that the service can be properly evaluated. Any complaints/praise should be recorded and followed up immediately. These should feed into the quality management system.

Workflow

Managing workflow in the histopathology laboratory involves consideration of efficiency, quality and

safety. The turnaround time for diagnosis continues to be a major concern and workflow must be established which provides the earliest possible diagnosis for patient treatment. Specimens must be moved from task to task in the most efficient manner possible. Laboratories have adopted continuous flow processing models which encourage small batch grouping and discourage bottlenecks and delays for specimens at any step. LEAN workflow management concepts have become popular in many manufacturing situations, and some laboratories have begun to embrace this method to improve workflow and employee satisfaction. LEAN thinking changes the focus of management from optimizing separate technologies and systems, to optimizing the flow of products and services through entire value streams which flow horizontally across technologies, assets and departments (for website see further reading).

Personnel management

One of the most important assets for a histopathology laboratory is its staff or personnel. Compared to any other laboratory specialty, the laboratory process in histopathology remains a manual procedure, from specimen receipt, through dissection (grossing), embedding, sectioning and staining. Many techniques are still reliant on skilled personnel rather than automation, and the laboratory manager must ensure that the department is staffed by an appropriate number of employees with the right level of skills to ensure that the process is robust, safe and cost-effective.

The laboratory manager is accountable for the service provided by the laboratory and should have the appropriate qualifications and experience to undertake this task. As well as being the lead technologist/scientist for the department, laboratory managers are usually responsible for recruiting appropriately and managing the human resource needs and professional direction of their staff.

All the staff should have comprehensive job descriptions so that they, their manager and supervisor know what is expected from them and to whom they are accountable. They should also have contracts which specify the terms and conditions of their employment.

The manager must ensure that there are appropriate numbers of staff with the required education, qualifications, training and competence to provide the service required. Managers must also ensure that the staff have access to further education as required to continue to keep up with the latest knowledge and techniques related to the service being provided. The competency of staff to do the tasks within their job description needs to be assessed at regular intervals. This, together with regular formal appraisals should ensure that staff are supported and provided with the requirements to fulfill their roles. The manager must also address any issues with discipline or excessive absence from work ensuring that the workforce team functions optimally.

Regular staff meetings should be held which involve all levels of staff to ensure that any new information is passed on, e.g. new procedures or updates related to the risk and quality management systems. These meetings allow staff to feed back any information they have, or raise queries and gives them access to supervisors or managers who they may not see during their routine day. LEAN management techniques encourage short staff meetings at the start of each day ensuring that any issues related to that day's work can be raised and planned for, e.g. staff absence, workload or other factors which may interrupt or disrupt the workflow.

Staffing the laboratory

Ensuring the right number and level of staffing depends on the manager having a good understanding of the volume and complexity of the work received. Good information systems are essential for recording and analyzing the volume of work performed in a laboratory, and for understanding trends in workflow and complexity.

Guidelines, e.g. those issued by the Royal College of Pathologists and the Institute of Biomedical Science in the UK, and by CLIA regulations in the US, advise what level of laboratory duties may be undertaken by which grade of staff. They have their own training and examination systems to enable consultant and postgraduate scientific staff to gain the qualifications they require. Scientific staff working in accredited laboratories in the

UK should be registered by the Health and Care Professions Council (HCPC), and in the US, they should be registered with the American Society for Clinical Pathology (ASCP). Some states in the US require laboratory personnel to be licensed. Licensing of technologists often requires a college degree and a specific exam or proof of ASCP certification, but requirements vary between state and specialty.

Once the level and complexity of the workload is known the workforce can be profiled to match its requirements, remembering that to be cost-effective, tasks not requiring registered or licensed scientists should ideally be performed by support staff.

Premises, equipment and materials

The laboratory environment and equipment must be fit for all the laboratory processes. Managers should ensure that there are adequate basic facilities for staff to do their jobs, e.g. rest and toilet facilities, adequate lighting, IT provision and space. There should also be enough space for equipment and storage. Equipment should be functional and regularly maintained for its safe use.

Staff must be trained and competent in their individual areas to use all of the equipment and materials in a safe and effective way. Display screen equipment (DSE) assessments must be carried out on all staff who regularly use computers.

Materials and equipment must be managed regarding stock control and servicing. Procurement policies should ensure that quality stock is purchased, is fit for purpose and value for money. Consideration should be given to whether to lease or purchase the equipment, as a lease contract may enable upgrades of equipment or software as part of the contract.

Financial management

Management of the departmental budget has become an important aspect of the laboratory manager's role. It is important to ensure that value for money is achieved in all aspects of service provision.

Financial consideration must be given when considering which tests to perform in house and which ones to send out, when recruiting and profiling the workforce, and when purchasing supplies and equipment. In the UK, most NHS Trusts have a cost saving target which will mean that managers will need to find ways to deliver a quality service, whilst producing a saving on the previous year's expenditure.

Writing business plans and justifications for new equipment requires a good understanding of budgets and financial flow.

Acknowledgments

We would like to thank Sheffield Teaching Hospitals NHSFT for their kind permission to adapt and use the risk severity and likelihood values from the Trust risk policy. We also wish to acknowledge Louise Dunk who contributed this chapter in the last edition.

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2 Chemical safety in the laboratory

Ada T. Feldman

Introduction

Improper handling of hazardous chemicals can produce significant health and/or physical harm. For many years countries issued their own national regulatory standards to assure employees were informed of the hazards in the workplace. The regulations and descriptions of hazards varied between countries. In 2003 the United Nations established the Globally Harmonized System (GHS) for the classification and labeling of chemicals. This GHS, adopted by the majority of the countries, has established a uniform system throughout the world for identifying chemicals and communicating their hazard information on labels and safety data sheets. Most of this chapter references the GHS (United Nations, 2015). The goal is to help one understand the chemical hazards and properly handle chemical substances in the histology laboratory. Whilst this chapter is written from the perspective of the United States and the appropriate code of federal regulation (CFR) numbers are referred to, it can be readily applied to any country.

Classifications of hazardous chemicals

The United Nations GHS provides detailed criteria to assign the hazard classification to a chemical substance. Each classification is also assigned a category number. The category number indicates the severity of the hazard: Category 1 represents the greatest hazard risk; higher numbers have lower risk factor. Classifications are divided into three major groups (Table 2.1). The GHS assigns to each classification standardized text/symbols which includes hazard statements, a signal word, a pictogram (Table 2.2), and precautionary statements.

A chemical can have multiple hazard classifications assigned. For example, the common clearing agent, xylene, is classified with multiple physical and health hazards. Table 2.3 shows the GHS information assigned to xylene (Fisher Scientific SDS, 2015).

Labeling of hazardous chemicals

In the USA, the Occupational Safety and Health Administration's (OSHA) Hazard Communication (29 CFR 1910.1200) requires the following elements to be placed on labels of hazardous chemicals:

1. Product name
2. Signal word (Danger or Warning)
3. GHS Hazard statement (classification)
4. GHS Pictograms
5. GHS Precautionary statements
6. Name, address and telephone number of the chemical manufacturer

Chemical manufacturers use the standardized GHS tests to classify a chemical and label it accordingly. If a laboratory mixes its own reagents, they would need to follow the same GHS criteria to determine the hazard classification of the reagent in order to label it correctly. This would be time consuming and/or expensive.

For laboratory prepared reagents, searching the internet for a manufactured equivalent reagent can provide the GHS classifications. Lot numbers, date of preparation (manufacturing), and person who prepared the reagent are other pieces of information that can be added to labels as required by a facility's protocols. In the USA, chemicals which are

Table 2.1 GHS hazard classifications

Health hazards	Physical hazards	Environmental hazards
Acute toxicity	Explosives	Acute aquatic toxicity
Skin corrosion/irritation	Flammable gases	Chronic aquatic toxicity
Serious eye damage/eye irritation	Flammable aerosols	
Sensitization	Oxidizing gases	
Germ cell mutagenicity	Gases under pressure	
Carcinogenicity	Flammable liquids	
Reproductive toxicity	Flammable solids	
Specific target organ system toxicity-single exposure	Self-reactive substances	
Specific target organ system toxicity-repeated exposure	Pyrophoric liquids	
Aspiration toxicity	Pyrophoric solids	
	Self-heating substances	
	Substances which in contact with water emit flammable gases	
	Oxidizing liquids	
	Oxidizing solids	
	Organic peroxides	
	Substances corrosive to metal	

manufactured and labeled in compliance with other regulatory agencies are exempt from the GHS label format (29 CFR1910.1200(b)(5)).

Exempted chemicals include beverage alcohols, drugs, medical devices, pesticides, tobacco products and cosmetics. In the pathology laboratory, fixatives, processing reagents and stains are considered medical devices and are thus exempt from the GHS labeling.

Working safely with hazardous chemicals

Whenever possible, hazardous chemicals should be replaced with safer alternatives, or volume usage should be reduced. Mercury is an example of a hazardous material which has been banned in many U.S. hospitals; thermometers and sphygmomanometers are now using mercury replacements. Pathology laboratories in turn have replaced the mercury-based fixatives (B-5, Zenkers) with zinc-based fixatives.

It may not be possible to eliminate all hazards from a laboratory but the risk factor can at least be reduced. Formaldehyde is classified as a carcinogen. Formaldehyde-free fixatives using glyoxal have equivalent preservation characteristics without the carcinogenic properties of formaldehyde (Anatech, Ltd., 1999). Similarly, the clearing agent xylene requires solubility criteria for tissue processing and staining. The various xylene substitutes (aliphatic hydrocarbons, limonene) provide the solubility criteria without the physical flammability hazard of xylene or the high multiple health hazards.

Safety data sheets (SDS)

Formerly known as Material Safety Data Sheets (MSDS), SDS are the primary source for identifying the hazards and proper handling requirements of a

Table 2.2 GHS hazard classifications and assigned pictogram (29 CFR 1910.1200 appendix C)

Flame over circle	Flame	Exploding bomb
		
Oxidizers	Flammables Self reactives Pyrophorics Self-heating Emits flammable gas Organic peroxides	Explosives Self reactives Organic peroxides
Corrosion	**Skull and crossbones**	**Gas cylinder**
		
Corrosives	Acute toxicity (severe)	Gases under pressure
Health hazard	**Exclamation mark**	**Environment**
		
Carcinogen Respiratory sensitizer Reproductive toxicity Specific target organ toxicity Mutagenicity Aspiration toxicity	Irritant Dermal sensitizer Acute toxicity (harmful) Narcotic effects Respiratory tract irritation	Acute aquatic toxicity Chronic aquatic toxicity

chemical. Prior to GHS there were multiple MSDS formats being used. Without a standardized format it was difficult to find information, especially during an emergency. The GHS established a standardized 16 section SDS format (Table 2.4). Sections 1-8 provide general information and also any information which may be needed quickly in an emergency (e.g. first-aid, spill, fire). Sections 9-12 have scientific data on which the classification is based and Sections 13-15 have regulatory information. Manufacturers are required to send SDS with the initial shipment of a chemical and whenever a change has been made in the SDS (29 CFR1910.1200(g)). Training programs should constantly be updated to assure dissemination of any new SDS information.

Section 1: Identification of the substance or mixture and of the supplier

This section provides the name of the product and must match the name found on the label. The section may list other common chemical names. For example, formaldehyde gas is also known as methanal, methylene oxide and methyl aldehyde. The Chemical Abstract Service # (CAS#) is a unique numerical code issued by the American Chemical Society to identify a chemical; the chemical formula may also be provided in Section 1. The EC# is also a chemical identifier for chemicals marketed in the European Union (EU). Some suppliers list the catalog number to make tracking and reordering easier. The recommended use of the chemical will also be provided. Generally, the latter will be

Table 2.3 GHS classification and information for xylene

Hazard classification	Flammable liquids, Category 3 Acute toxicity-dermal, Category 4 Acute toxicity-inhalation (vapors), Category 4 Skin irritation, Category 2 Serious eye damage/eye irritation, Category 2 Carcinogenicity, Category 2 Specific target organ toxicity-single exposure, Category 3 Specific target organ toxicity-repeated exposure, Category 2 Aspiration toxicity, Category 1
Signal word	Danger
Pictograms	
Hazard statement	Flammable liquid and vapor Harmful in contact with skin Causes skin irritation Causes serious eye irritation May cause respiratory irritation May cause drowsiness or dizziness Suspected of causing cancer May be harmful if swallowed and enters airways May cause damage to organs through prolonged or repeated exposure
Precautionary statements	Prevention, response, storage and disposal statements

generic with such listing as 'laboratory chemical'. The supplier's contact information and an emergency telephone number are also provided. When an emergency telephone number is identified as CHEMTREC or CHEMTEL, that number is only to be used for a transportation emergency response and not for a medical emergency or a laboratory spill cleanup.

Section 2: Hazards identification

This section will provide the classification(s) of the chemical. In addition, all the label's elements will be found in this section of the SDS (signal word, pictogram(s), hazard statements, precautionary statements).

Table 2.4 GHS safety data sheets format

Section 1	Identification of the substance or mixture and of the supplier
Section 2	Hazards identification
Section 3	Composition and information on ingredients
Section 4	First-aid measures
Section 5	Fire-fighting measures
Section 6	Accidental release measures
Section 7	Handling and storage
Section 8	Exposure controls and personal protection
Section 9	Physical and chemical properties
Section 10	Stability and reactivity
Section 11	Toxicological information
Section 12	Ecological information
Section 13	Disposal considerations
Section 14	Transport information
Section 15	Regulatory information
Section 16	Other information

Section 3: Composition and information on ingredients

The chemical name as well as the common name will be listed for all components which contribute to the chemical hazard. Any hazardous impurities or stabilizers will also be included. An example of a hazardous stabilizer is found in 37% formaldehyde. Formaldehyde will polymerize to form solid paraformaldehyde and methanol, between 7-14%, is added to the aqueous solution to inhibit this reaction. Therefore, methanol, a hazardous chemical, is listed in the SDS as part of the composition of 37% formaldehyde. Besides the name, CAS#, EC# and concentration/concentration ranges are listed. A statement is required from the manufacturer when the specific chemical identity and/or exact percentage of composition has been withheld as a trade secret (29 CFR1910.1200(i)(1)).

Table 2.5 Standard first-aid treatment	
Eye contact	Rinse immediately with plenty of water for at least 15 minutes. Obtain medical attention.
Skin contact	Remove any contaminated clothing. Wash off immediately with plenty of water for at least 15 minutes. Obtain medical attention.
Inhalation	Move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Obtain medical attention.
Ingestion	Do not induce vomiting. Obtain medical attention.

Section 4: First-aid measures

This section contains universal/standard treatment measures for when an exposure has occurred (Table 2.5). The directions are intended for immediate medical care by untrained responders or those that have taken a basic first-aid course. An exposure to hydrochloric acid can have symptoms which vary from a simple irritation to severe burns. As indicated by the adage 'The dose makes the poison', this variability of symptoms results in the basic instructions ending with 'obtain medical attention'. Professional medical personnel are best able to determine the degree of injury and the correct way to remedy the specific exposure. GHS formatted SDS requires first-aid procedures to be given for the possible routes of exposure: inhalation, skin and eye contact, ingestion. The symptoms for both acute and chronic exposure symptoms must be provided. Every work area should have a basic first-aid kit containing at least adhesive bandages, sterile gauze pads and adhesive tapes to dress a wound prior to obtaining advanced medical attention.

Eye washes can be plumbed or self-contained. The American National Standards Institute (ANSI) recommends that eyewashes deliver three gallons of tepid water per minute for 15 minutes (ANSI, 2014). Plumbed eyewashes are connected to a source of tap water and have the advantage of delivering large volumes of water. The disadvantage of plumbed equipment is monitoring the temperature and possibly the quality of the tap water. Self-contained eyewashes store pH balanced rinse solutions which are more comfortable for rinsing the eyes. Microorganisms can grow in eyewash stations (stagnant water) if the lines are not flushed on a regular basis. In addition, the self-contained eyewashes must be refilled/replaced according to manufacturer's recommendations. Improper maintenance of the eyewash can provide additional health hazards and therefore cause additional harm to the eyes beyond the chemical exposure (OSHA Info sheet, 2015). In the USA, the Formaldehyde Standard requires a laboratory to have eyewash stations within the immediate work area (29 CFR 1910.1048(i)(3)). In addition, workplaces which expose workers to corrosives are required to have eyewashes in the immediate area (29 CFR 1910.151). Equivalent standards are required for shower equipment.

Section 5: Fire-fighting measures

The specific type of extinguishing media to be used in case of a fire will be found in this section. Work areas generally have dry chemical multipurpose fire extinguishers for handling all types of fires. Table 2.6 summarizes the types of extinguishers to use with different classes of fires (Fire Equipment Manufacturers' Association, 2016). Laboratories should routinely inspect their areas for fire risks and have appropriate extinguishers readily available. OSHA requires travel distance to a fire extinguisher be no more than 100 feet. When more than 5 US gallons of flammable liquid are located in a work area a fire extinguisher must be provided within 50 feet (29 CFR1926.150). The fire safety program of a facility should instruct the proper use of a fire extinguisher. Local fire authorities or hospital fire officers provide fire extinguishing training and in most institutions annual mandatory fire training programs occur. Besides the correct extinguisher to use, this section will also warn one of the inappropriate fire extinguishers. Special instructions are given for firefighters of any specific hazards resulting from a chemical fire and the required fire-fighting protective equipment which should be worn.

Table 2.6 Fire extinguishers, their classification and associated types of fires

Types of extinguisher		Types of fire
Water and foam	Class A	Combustibles such as paper, wood, trash
Carbon dioxide	Class B	Flammable liquids: alcohol, xylene
	Class C	Electrical equipment
Dry chemical	Class A	Combustibles such as paper, wood, trash
	Class B	Flammable liquids: alcohol, xylene
	Class C	Electrical equipment
Wet chemical		Kitchen fires
Dry powder	Class D	Metal fires
Water mist	Class A	Combustibles such as paper, wood, trash

Section 6: Accidental release measures

One must know the hazard(s) of a chemical/mixture in order to know how to properly clean up any spillage. This section provides information on the specifics of the procedure for a spillage cleanup. It is required reading so that the laboratory is prepared with all the specific spill cleanup supplies (personal protective equipment [PPE], absorbents, neutralizers). Depending upon an institution's spill response program, either outside assistance and/or trained employees will perform the cleanup.

Most laboratory spillages involve small quantities and with the proper training, PPE and cleanup supplies they are easy to remedy. Evaluating the risk of a spill is an important element in the cleanup. It is more than the size of a spill that will determine the cleanup procedure. The characteristics of the chemical and ventilation play a major role. In a well ventilated area, a few hundred milliliters of spilled alcohol might be considered a small spill and only require absorbent materials to contain and remove it. However, a few hundred milliliters of formaldehyde in a poorly ventilated room could exceed exposure limits and produce a higher health risk requiring a different mode of cleanup involving respirators.

A spill response procedure must be developed to cover every chemical used in the laboratory (American Chemical Society, 1995). The written procedure must document evacuation routes and alarms in case they are needed. All spill cleanup materials and methods required for each chemical (group) must be included in the spill response document.

General procedures for a spill cleanup include (American Chemical Society, 1995):

1. Evaluate the risk to determine if trained employees can neutralize the spill or if outside assistance is needed.
2. Evacuate people from the area if required.
3. All individuals performing any cleanup must wear appropriate PPE: goggles, gloves, shoe covers, aprons, and respirators. The PPE must be chemically resistant for the material being cleaned up.
4. Prevent the spread of any vapors by increasing ventilation. Turn off all ignition sources if cleaning up a flammable liquid.
5. Control the spread of a liquid by using absorbent materials. Absorbent socks, booms, pillows and pads are convenient and less messy than vermiculite or kitty litter. Special pads are available for formaldehyde which neutralizes the chemical and absorbs the formaldehyde vapors.
6. Absorb the liquid. Neutralize if possible. Acids are easily neutralized with sodium bicarbonate; bases with citric acid. The neutralizing procedure is exothermic and a bubbling reaction is normal.
7. Collect contaminated materials (booms, pads, neutralized residue, contaminated PPE). If cleaned up material is hazardous, properly label the

contaminated items and dispose of as hazardous waste. Non-hazardous (neutralized material) may be disposed in regular trash.

8. Wash down the contaminated area with suitable cleaning solutions. Generally, soapy water is used. Non-aqueous spills will need cleanup solvents which are not water based. Continue ventilating the area to rid it of any vapors resulting from the spill.
9. Perform all notifications and required reports.

Special cleanup procedures are required for metallic mercury. Metallic mercury from a broken thermometer can disperse toxic vapors. Generally, the mercury can be seen and collected into larger droplets with a damp cloth and collected for disposal. If metallic mercury is used in the laboratory, special mercury absorbing sponges should be part of the spill kit. A special mercury vacuum is also an option for a metallic mercury spill cleanup.

In addition to the implementation of a chemical spill cleanup program, the lab should also have an equivalent program for biohazards. Detailed information for handling biohazard spills are beyond the scope of this chapter. However, the general protective steps listed for a chemical cleanup should be followed. Decontamination procedures and disinfectants will vary. A freshly prepared 10% bleach solution is commonly used to disinfect solid surface areas.

Section 7: Handling and storage

This section will provide the environmental conditions for safe storage of a chemical whilst also maintaining the integrity of the chemical. In addition, the physical incompatibilities (e.g. heat, light, water) for improper storage are also provided. Room temperature storage is recommended for most laboratory chemicals. Generally, chemicals should not be exposed to heat or direct sunlight. For example, hematoxylin stains will break down faster if stored in direct sunlight. Acetic acid should be stored above 61°F (16.6°C) to prevent it from freezing. Its characteristic of forming ice crystals at such high temperatures is where it acquired the common name, glacial acetic acid.

Chemicals will be identified as to whether they require storage in special cabinets due to flammability or corrosive characteristics. Flammables and corrosives should each be stored in approved storage cabinets. OSHA limits 60 gallons inside a safety cabinet for Class I & II flammables (29 CFR1910.106 (d)(3)(i & ii)). Ethyl alcohol, isopropyl alcohol and xylene are used in large volumes in histology and are all Class I flammables. Local fire departments often set their own limitations. Explosion-proof laboratory refrigerators should be used when flammables need to be refrigerated. A household refrigerator/freezer should never be used to store flammable materials.

Storage incompatibilities for chemicals are provided in Section 10 of the SDS. Chemicals should never be stored alphabetically except when grouped within the same hazard class. Acids should be stored by themselves; segregation of the different acid groups from each other will also be noted in this section of the SDS.

Chemicals should be stored in a location, e.g. a shelf, below head level. This prevents any chance of spillages from contaminating the head while reaching for chemicals. Storing chemicals on secondary trays allows the collections of miscellaneous drips or containment in case of a bottle breakage. It is good practice when transporting a bottle of concentrated acid or base, even across the room, to place it in a secondary container such as plastic bucket with an appropriate lid.

Section 8: Exposure controls/personal protection

Exposure control limits are issued by governmental agencies or are recommended by the chemical manufacturer. In the USA, permissible exposure limits (PELs) are issued by OSHA and represent the amount a worker may be exposed to during a time period without any adverse health effects. OSHA has two standard PELs.

Time weighted average (TWA) represents the exposure limit averaged over an eight-hour time period. A TWA is appropriate in a manufacturing facility where chemical exposure is constant during a work shift. A TWA is not practical in a laboratory environment where there could be a high exposure for a very short time period and none for the rest of the day.

The short term exposure limit (STEL) does take a laboratory environment into consideration. A STEL

measures the exposure for a 15-minute time frame. In the USA, OSHA has issued PEL for formaldehyde (vapors) as TWA = 0.75 ppm and STEL = 2.0 ppm (29 CFR 1910.1048(c) (1) & (2)). The American Conference of Governmental Industrial Hygienists (ACGIH) also issues exposure limits identified as threshold limit values (TLV). Their data closely align with OSHA's PEL. OSHA and ACGIH do not have exposure limits for all of the thousands of chemicals manufactured. Therefore, many chemicals have exposure limits issued by the manufacturer's research and development data. The SDS will identify the source of the exposure limit with the name of regulatory agency, organization or manufacturer.

As well as providing any exposure limits, Section 8 also provides engineering controls to control/reduce exposure. General laboratory ventilation systems should have an adequate exchange rate to ensure vapors do not accumulate or recirculate through the building. Laboratory ventilation is recommended to be 6 to 12 room air changes per hour (National Research Council Committee, 2011). Chemical hoods must be used when general room ventilation is inadequate and the release of odoriferous vapors can occur if used out in the open. Styles of exhaust hood vary and the choice must take into considerations the chemicals used, how it will be used and available air exchange/engineering controls. An explosion-proof chemical hood must be used with flammable chemicals. Chemicals or equipment must never be stored in a hood because they could disrupt the air flow. When working with chemicals, they should be 6 inches (15 cm) inside the hood with the sash in the lowest possible position. The face velocity of the chemical hood should be checked on a regular basis. For general chemicals, a face velocity between 80-100 feet per minute (fpm) is recommended. Face velocities of 100-120 fpm are recommended for chemicals of very high toxicity (National Research Council Committee, 2011).

When engineering controls or work practices do not prevent the exposure to hazardous chemicals, personal protective equipment (PPE) is required. PPE should be readily available at all times and stored in a familiar location. The laboratory's safety hygiene plan should document which procedures (e.g. changing solvents on tissue processor, loading tissue cassettes into baskets) require which type of PPE. As with spill cleanups, PPE must be of a material and style appropriate for the type of chemical (e.g. acids, petroleum solvent, aqueous), nature of exposure (e.g. liquid, vapors) and level of exposure (above or below PEL).

Eye protection

Safety glasses are not adequate protection when using chemicals. They are designed to protect eyes from flying objects. Splashes and dust can still reach the eyes when using safety glasses, even those with a wraparound style. Goggles provide a secure shield on the face and are required especially when handling liquids. Non-vented goggles are the best to use because they prevent vapors and dust from reaching the eyes. Long term usage of goggles may result in fogging. Anti-fogging coatings on the goggles are available as well as wipes to reduce fogging. Face shields can be used as secondary protection with safety glasses or goggles. Face shields should not be worn as the only source of PPE for the eyes.

Skin protection

'One size fits all' does not apply when choosing a glove to prevent exposure to a chemical. Glove material and required dexterity for the required task must be taken into consideration when selecting a glove. The glove material will determine the compatibility with the chemical. Chemical compatibility charts for gloves are available on the internet to help choose the correct protective glove (Ansell Healthcare, 2008; North, 2010). Selection of a glove's material must take into consideration its characteristics of degradation time, breakthrough time and permeation rate of the chemical being handled. Manufacturers can vary glove thickness. Therefore, a glove made of the same material from different manufacturers will vary in the degree of protection. Whenever possible check with the manufacturer for their glove test data.

Latex gloves which provide protection against pathogens provide minimal protection against chemical exposure, especially organic solvents like xylene.

Latex gloves are also associated with allergy reactions in a significant part of the population, thereby limiting their value. Nitrile and neoprene gloves can be used as an alternative for protection from acids and organic solvents and are more regularly used now.

Aprons, protective suits and footwear coverings should also be chosen based on the risk level encountered. Measuring out 1 ml of acetic acid needs minimal protection of gloves and a disposable apron, while cleaning up a large acid spill generally requires a protective suit and footwear/boots. Laboratory coats can have protective finishes which are fluid resistant. These are generally acceptable for very small volumes and are designed more as a protective layer for stains and spills from reaching underlying clothing.

Respiratory protection

Particle (dust) masks and respirators are the two major types of respiratory protection used in the laboratory. Particle masks can be disposable or reusable. The National Institute of Occupational Safety and Health (NIOSH) rates particle masks, assigning them a letter and a number to designate intended use and capacity respectively. The letter N indicates the mask cannot filter oil-based particles; R represents it is oil resistant up to 8 hours; P represents the mask is oil proof beyond 8 hours. The number represents the percentage of one-micrometer particles which can be filtered from the air. Most commonly used in the hospital is an N95 particle mask. It will filter 95% of 1 micrometer sized, non-oil-based airborne particles from the air (Today's Homeowners, 2016). While an N100 provides more particle protection, all particle masks are not suitable for use with chemical vapors.

Respirators with chemical cartridges must be worn when handling chemicals releasing vapors above the PEL or when the user is susceptible to the gases/vapors. Respirators may be full face or half-mask. The half-mask covers only the mouth and nose so gas-proof goggles must be used with them to protect the eyes from exposure to the vapors. Cartridges are chosen based on the chemical being used (e.g. formaldehyde, organic vapors, ammonia, acid gases). Cartridges contain activated carbon treated to absorb the vapors and are color coded to indicate the contaminant it can filter, e.g. white for acid gases, black for organic vapors, and olive for formaldehyde. Combination cartridges are available which can filter multiple classes of gases, particles, and vapors (3M Personal Safety Division). When respirators are required to be used a respiratory fit testing program must be established. The respiratory fit testing assures the wearer is capable of wearing a respirator since breathing through a respirator is more difficult than breathing normal air. People with inadequate lung capacities or eyesight problems may not be able to work using a respirator. Also the respiratory fit testing program must show the user how to wear the respirator correctly.

Ingestion protection

Laboratories which are accredited by the College of American Pathologists (CAP) are required to have a written policy which prohibits eating, drinking, smoking, application of cosmetics, and mouth pipetting in the laboratory (CAP Checklist, 2014). Such requirements are common in all laboratories and help prevent any contamination of these items which are ingested or applied to the mouth area. Washing your hands and face after handling chemicals and after removing gloves is essential in a medical laboratory. This simple measure will prevent ingestion of chemical vapors and microbes which could have settled on the skin.

Section 9: Physical and chemical properties

There are 18 physical/chemical properties which are required (29 CFR1910.1200 Table D.1) to be listed in the SDS (Table 2.7). If no data are available or are not applicable, then the SDS will state so. Many of these properties are used to determine the chemical classification(s).

In the histology laboratory many of these properties can provide additional information on the handling of the chemical. When using an unfamiliar solvent for the first time, the appearance and odor data can confirm that the physical characteristics are correct. Histological stains have pH ranges that produce optimal staining results. When the pH is out of the range, staining results will be abnormal (non-specific, too light, too dark). Knowing

Table 2.7 Physical and chemical properties and application

Property	Application
Appearance (physical, state, color)	Troubleshooting
Odor	Troubleshooting
Odor threshold	Troubleshooting
pH	Troubleshooting
Melting point/freezing point	Storage conditions
Initial boiling point and boiling point range	Storage conditions
Flash point	Storage conditions
Evaporation rate	Important information for mounting media
Flammability (solid, gas)	Storage conditions
Upper/lower flammability or explosive limits	Storage conditions
Vapor pressure	Ventilation information
Vapor density	Ventilation information
Relative density	Ventilation information
Solubilities	Cleanup information
Partition coefficient: n-octanol/water	Cleanup information
Auto-ignition temperature	Storage conditions
Decomposition temperature	Storage conditions
Viscosity	Important information for mounting media

the correct pH value is helpful in troubleshooting. Multiple properties are listed to provide the specific temperatures to prevent melting or freezing of the chemical. Solubility data assist in cleanup information by informing whether the chemical is water or oil soluble.

Section 10: Stability and reactivity

Chemicals and mixtures not only have health hazards but also, if improperly stored or mixed, can produce physical hazards such as the release of toxic vapors or an explosion. For example, mixing acetic acid with bleach can generate toxic chorine gas. Some chemicals are self-reactive under heat or pressure.

Picric acid, used in Bouin's fixative, is stable in liquid form (greater than 30% water), but the dry form is sensitive to friction, heat and shock, and is explosive. When storing a picric acid solution checks should be performed on a monthly basis to ensure that the quantity of water is maintained to prevent it from drying out (McGill University, 2016). When pouring liquid picric acid one should wipe the inside edges of the cap and the rim of the bottle free of any liquid to prevent any drying of picric acid solution. The cap should be kept loose to prevent any chance of even the smallest explosion due to the friction when turning the cap.

Hazardous decomposition is the term used to describe the breakdown of an unstable molecule into smaller molecules. This breakdown can result in an explosion, fire or release of vapors. Such chemicals are rarely used in the histology laboratory. This section of the SDS identifies such chemicals or mixtures. Section 7 also provides the safe handling and storage of such chemicals.

Section 11: Toxicological information

This section contains the supportive data used to classify the health hazards of the chemical. Numerical measures of toxicity such as LD_{50} and LC_{50} are provided. LD_{50} is the lethal dose in which 50% of the tested population died; LC_{50} is the lethal concentration (used for vapors) in which 50% of the tested population died. Specific symptoms observed for the inhalation, ingestion, and skin and eye contacts are provided. Toxicology information is provided for both long-term and short-term exposure as well as the delayed and immediate effects. For example, skin exposure to acids produces an immediate effect from redness (short exposure) to severe burns (long-term exposure). By contrast, breathing in asbestos bodies over a long term has a delayed reaction of possible severe lung disease. This section of the SDS can be used to assist medical professionals during medical treatment. The listed numerical data are usually based on animal studies, where experiments are carefully controlled so that the exposure is only to the one chemical. It has been difficult to assign inhalation dose and effects in human occupational studies since most workers

are exposed to multiple chemicals in their work and private life.

Section 12: Ecological information

Whilst discussion is not warranted for this safety chapter, this section of the SDS has important information to help determine disposal options.

Section 13: Disposal considerations

Guidelines are given for disposal of a chemical. However only the applicable governmental agency has the authority to provide the disposal method. Each country has developed its own environmental laws and regulations. In the USA, the Environmental Protection Agency (EPA) oversees disposal controls. However, each of the state and local municipalities in the USA are able to issue more stringent rulings than the federal government. Therefore, each user must dispose of any chemical in accordance with their local governmental directives.

Drain disposal is the easiest disposal option. One must get permission from their public-owned wastewater treatment plant to assure that drain disposal will not affect the functions of the facility. One step in the treatment process involves bacteria and it is important that the drain-disposed chemical is not toxic to the bacteria. It is beneficial when the chemical being disposed is water soluble, since the toxic effect is reduced through standard water dilution whilst traveling through the pipe systems. Wastewater treatment plants vary in their capabilities, and therefore the volume and classification of the chemical being drain disposed will determine whether permission is granted. Most hospital solid and liquid wastes are discarded with the services of a licensed waste hauler. Work with the waste hauler to properly segregate and label the waste material.

Recycling

Recycling is a viable option to reduce waste volumes and disposal costs in the histology laboratory. Distillation recycling systems for alcohol and xylene/xylene substitutes offer great cost-savings on these two high-volume solvents. The capital expense for the equipment will be offset by the reduced waste hauling expense. These systems are able to deliver the same purity of the original product purchased. Recycling units are programmed with the boiling point of the solvent. During the distillation process, the solvent is boiled and the vapors are collected for use. Whilst the recycled material for xylene can be used in the same capacity as any unused clearant, recycled alcohols have limitations. The concentration of recycled alcohol is approximately 95% due to the azeotrope properties of alcohol and water. Therefore, distilled alcohol cannot be used in procedures which call for 100% alcohol.

Formalin distillation recyclers use the same principle of the alcohol and xylene distillation units. However, the action of boiling can destroy formaldehyde. It is standard procedure to test the recycled formaldehyde to assure the concentration is 10%. Commercial assay test kits make this easy to determine. During formaldehyde distillation the buffering salts are not recycled. Therefore, it is necessary to re-buffer the recycled formalin before use.

Sometimes the recycled material may smell fishy. This is due to dissolved serum proteins in the waste solvents from the tissue processor. During exposure to the high temperatures required for distillation the serum proteins are broken down into odoriferous amines (producing the fishy smell). Keeping the distillation unit properly cleaned can easily prevent this fishy smell. Manufacturers of distillation units have recommended cleaning procedures.

Filtration recycling units do not use distillation but instead use absorbents to remove the color and solid materials from the waste solvent. Water from the tissues during processing, or carryover from the staining procedure, contaminates formalin and/or alcohols reducing the solvents' concentrations. The excess water is not removed by filtration and the concentration of the recycled material stays the same as the starting (waste) material. When using filtration recycling units, chemical testing of the formalin and an alcohol hydrometer needs to be used to determine the concentration of the recycled material and its applicable use.

Section 14: Transport information

The classification procedure to determine the hazards classification of a chemical for transportation is

based upon regulations written by the Department of Transportation (DOT) (USA) and the International Air Transportation Agency (IATA). The transportation classification text uses terminology such as proper shipping names, packing groups and packing classes. Both GHS and transportation standards use pictograms but with different criteria. Transportation regulations only identify a chemical as corrosive when they react with skin or metal; a chemical used in the workplace which is corrosive to the eyes will have a GHS corrosive pictogram on the bottle label but the shipping box containing the bottle may not have the same pictogram. Therefore, do not use any of the information found in the transportation section (e.g. proper shipping name, transportation diamonds) to identify the hazards which are encountered in the workplace.

Section 15: Regulatory information

The SDS is designed to provide information for occupational safety. This section will list all other national and state regulatory standards (environmental, transportation, consumer protection) which the chemical/mixture must comply with.

Section 16: Other information

This section will minimally have the SDS issue and/or revision date. Many SDS will have the information for the NFPA hazard diamond (composed of 4 smaller diamonds). Each smaller diamond is uniquely colored for flammability (red), health (blue), instability (yellow) and special hazards (white). The hazard risk is indicated with a number in each smaller diamond ranging between zero (least hazardous) and four (most hazardous). The GHS classification category numbers however, represent hazards inversely from the NFPA numerical system. GHS category 1 represents the most hazardous; category 4 indicates least hazardous. It is therefore important not to use the GHS category numbers to complete NFPA diamonds.

The NFPA standards (National Fire Protection Association, 2012) identify the hazard risks which are experienced during a fire or spill emergency and are not to be used for occupational exposure. Despite the intended use and how the hazard risks have been assigned, many hospitals adhere NFPA diamonds onto their chemical bottles. The NFPA diamonds are actually to be attached to the outside of buildings or areas to warn emergency responders of the possible hazards they may encounter.

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3 Light microscopy

John D. Bancroft

Introduction

This is an introduction to the theory of light microscopy. The subject is dealt with in more depth in the previous editions of this book and further information may be found in dedicated texts to the subject.

The light microscope is an essential part of the histopathology laboratory as it is the device with which histological preparations are studied. The designs and specifications of modern microscopes vary widely, but the basic principle is the same as the original simple microscope which used sunlight as its light source (Fig. 3.1). Electric bulbs or light emitting diodes (LEDs) are now used to produce a beam of light which is focused on the tissue section or sample, and then the transmitted light passes through a set of objectives, along the tube and through the eyepiece into the eye of the microscopist.

Fig. 3.1 A standard modern light microscope.

The lens system of the light microscope allows the eye to see an image of the target tissue at varying magnifications depending on the objectives used. The varying lenses seen in the modern microscope are present within the substage condenser below the slide, as well as above it. The additional objective lenses above the sample can be brought into position depending on the tissue magnification required. The objectives are usually mounted in a rotating disc and are brought into alignment with the main body tube of the microscope to select higher or lower magnifications.

The different magnifications required are achieved by altering three variables; firstly, the angle at which the light strikes the lens, the angle of incidence; secondly, the curvature of the lens and finally, the density of the glass or refractive index (RI). Parallel light entering a lens from a small object is brought to a sharp focus at a point behind the lens, then the eyepiece allows a magnified real image to be formed below the eyepiece (Fig. 3.2). This is the basic principle of light microscopy.

Light and its properties

Visible light occupies a narrow portion of the electromagnetic spectrum and can be detected by the human eye, although the full spectrum extends from radio and microwaves through to gamma rays. Electromagnetic energy is complex, having both wave and particle-like properties.

It is common practice to illustrate the light in the electromagnetic spectrum as a sine wave. The distance from one wave peak to another is the wavelength of light (Fig. 3.3).

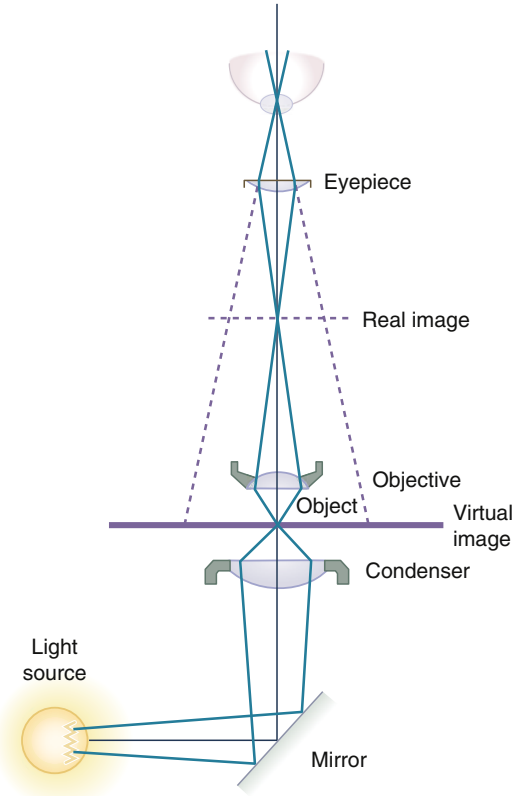

Fig. 3.2 The light ray path through the microscope. The eye sees the magnified virtual image of the real image, produced by the objective.

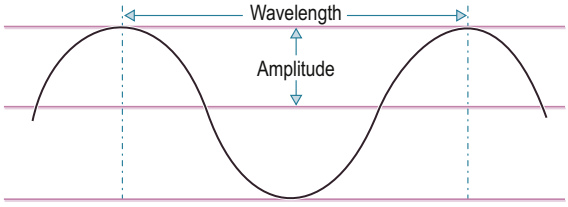

Fig. 3.3 Representation of a light ray showing its wavelength and amplitude.

Light with a single wavelength is monochromatic, but the majority of light sources are composed of many different colors and wavelengths which are refracted in different directions. The panspectral distortion which can occur to an image can be corrected by different types of lenses within a microscope.

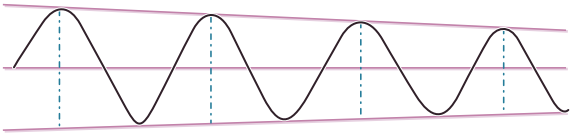

Fig. 3.4 The amplitude, i.e. brightness diminishes as light gets further from the source due to absorption into the medium through which it travels.

The human eye responds to a complex mixture of light of different wavelengths and when this approximates to the mixture of light derived from the sun, it is known as 'white' light. By definition, white light is a mixture of light which contains a percentage of wavelengths from all of the visible portions of the electromagnetic spectrum. One measure of the mixture of light given off by a light source is *color temperature*. The higher the color temperature, the closer the light is to natural daylight derived from the sun.

Light sources produce light in all directions and usually consist of a complex mixture of wavelengths which define the color temperature of the light source. Some sources, e.g. tungsten filament and xenon lamps provide a relatively uniform mixture of wavelengths, although of different amplitudes or intensities. Others, e.g. mercury lamps, provide discrete wavelengths scattered over a broad range, but with distinct gaps of no emissions between these peaks.

Most light sources are non-coherent, but standard optical diagrams draw light rays as straight lines even though the actual light is emitted from the source in all directions. Another property important in understanding microscope optics is that some of the light is absorbed by the media (lens and air) through which it passes (Fig. 3.4). This produces a reduction in the amplitude, or energy level, of the light. The media can also have an effect on the actual speed of the light passing through the microscope, this is known as *retardation*.

Retardation and refraction

Media through which light is able to pass will slow down or *retard* the speed of the light in proportion to the density of the medium. The higher the density the greater the degree of *retardation*. Rays of light entering

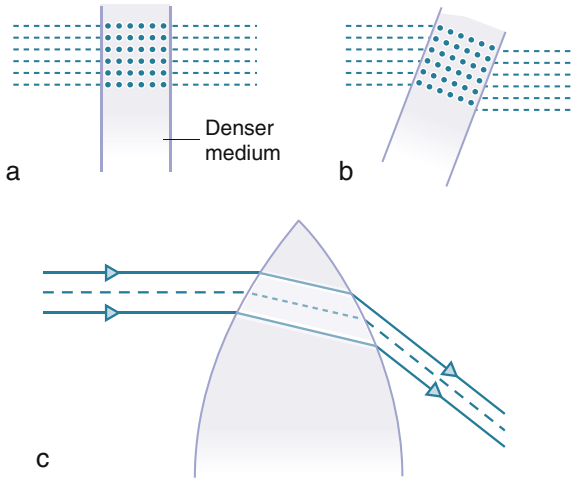

Fig. 3.5 **(a)** Light rays passing from one medium to another at right angles to the interface are slowed down equally, i.e. retarded. **(b)** Rays passing at any other angle to the interface are slowed down in the order that they cross the interface and are deviated, i.e. refracted. **(c)** Rays passing through a curved lens are retarded and refracted.

a sheet of glass at right angles are retarded in speed but their direction is unchanged (Fig. 3.5a). When light enters the glass at any other angle, a change of direction also occurs and this is called *refraction* (Fig. 3.5b). A curved lens will exhibit both retardation and refraction (Fig. 3.5c) and this is governed by:

- The angle at which the light strikes the lens, the *angle of incidence.*
- The density of the glass, its *refractive index.*
- The curvature of the lens.

The angle by which the rays are deviated within the glass or other transparent medium is the *angle of refraction* and the ratio of the sine values of the angles of incidence (i) and refraction (r) gives a figure known as the *refractive index* (RI) of the medium (Fig. 3.6a). The greater the RI, the higher the density of the medium. The RI of transparent substances is important in the computation and design of lenses, microscope slides, coverslips and mounting media. Air has a refractive index of 1.00, water 1.30 and glass has a range of values depending on the type, mostly averaging 1.50.

Usually, light passing from one medium into another of higher density is refracted towards the

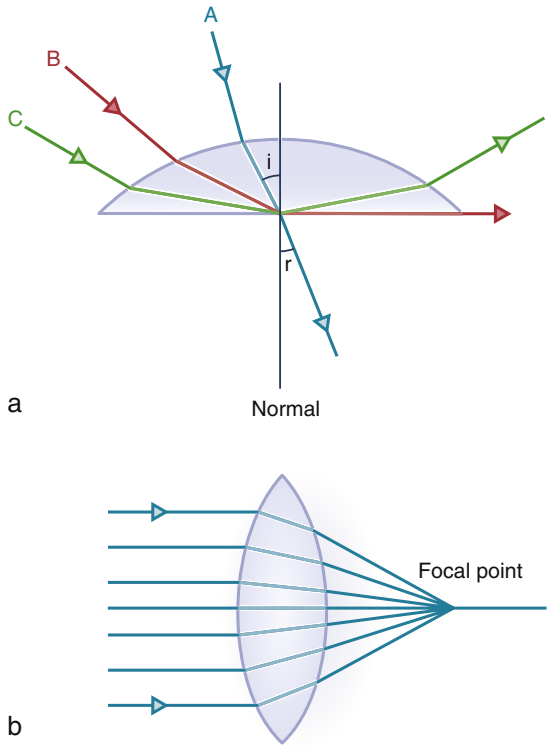

Fig. 3.6 **(a)** Light ray A shows the angles of incidence (i) and refraction (r). Ray B, entering the lens at an increased angle of incidence, is lost through the edge of the lens. Increasing the angle of incidence further, Ray C shows total internal reflection. **(b)** Parallel rays entering a curved lens are brought to a common focus, the focal point.

normal, and when passing into a less dense medium it is refracted away from the normal. The angle of incidence may increase to the point where the light emerges parallel to the surface of the lens – beyond this angle of incidence *total internal reflection* will occur, and no light will pass through (Fig. 3.6a).

Image formation

Parallel rays of light entering a simple lens are brought together by refraction to a single point, the principal focus or *focal point*, where a clear image will be formed of an object (Fig. 3.6b). The distance between the optical center of the lens and the principal focus is the *focal length*. A lens has an additional pair of points, one either side of the lens, called *conjugate foci*, and an object placed at one will form a

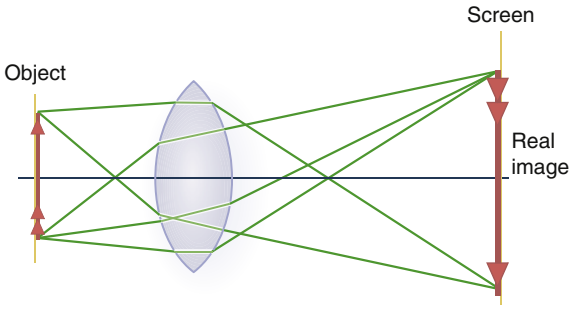

Fig. 3.7 A real image is formed by rays passing through the lens from the object, and can be focused on a screen.

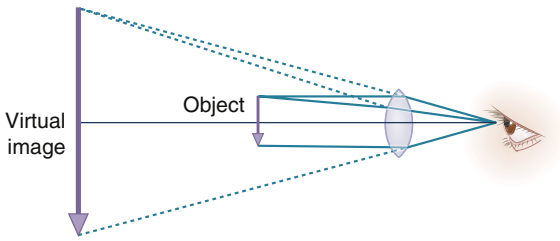

Fig. 3.8 A virtual image is viewed through the lens but appears to be on the object side of the lens.

clear image on a screen placed at the other. The conjugate foci vary in position – as the object is moved nearer the lens the image will be formed further away, at a greater magnification, and inverted. This is the *real image* and is formed by the objective lens of the microscope (Fig. 3.7).

If the object is placed nearer the lens, within the principal focus, the image is formed on the same side as the object and is enlarged, the correct way up, and cannot be projected onto a screen. This is the *virtual image* (Fig. 3.8) and is formed by the eyepiece of the microscope from the real image projected by the objective. This appears to be at a distance of approximately 250mm from the eye, near the object stage level. This may be illustrated as in Fig. 3.2 with the formation of both images in the upright compound microscope.

Image quality

White light is composed of all the visible spectral colors and on passing through a simple lens, each wavelength is refracted differently – blue is

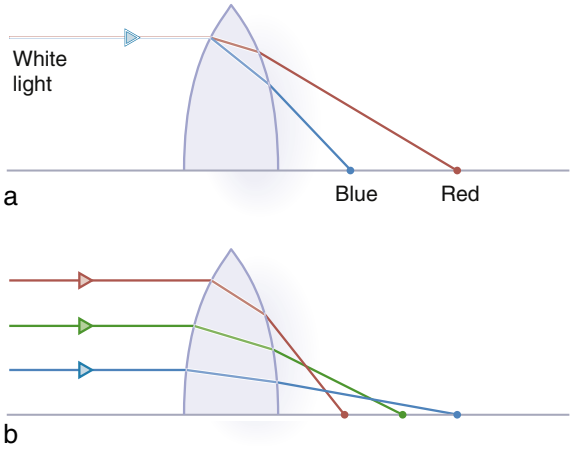

Fig. 3.9 **(a)** Chromatic aberration. **(b)** Spherical aberration.

brought to a shorter focus than red. This lens defect is *chromatic aberration* (Fig. 3.9a) and results in an unsharp image with colored fringes. It is possible to construct compound lenses of different glass elements to correct this fault. An *achromat* corrected for blue and red produces a secondary spectrum of yellow and green; this in turn can be corrected by adding more lens components, the more expensive *apochromat*.

Microscope objectives of both achromatic and apochromatic types are usually overcorrected for longitudinal chromatic aberration and must be combined with matched compensating eyepieces to form a good quality image. This restriction on changing lens combinations is overcome by using chromatic, aberration free (CF) optics which correct for both longitudinal and lateral chromatic aberrations, and remove all color fringes. This is useful for fluorescence and interference microscopes.

Other distortions in the image may be due to coma, astigmatism, curvature of field and spherical aberration; they are due to the lens shape and quality. *Spherical aberration* is caused when light rays entering a curved lens at its periphery are refracted more than those rays entering the center of the lens and are not brought to a common focus (Fig. 3.9b). These faults are corrected by making lenses of different glass components, e.g. fluorite, and of differing shapes.

The components of a microscope

The light source

A progression of light sources has been used from sunlight, oil lamps, low-voltage electric lamps and now LEDs. The latter operate via a transformer and can be adjusted to the intensity required.

Condensers

Light from the source is directed into the first major optical component, the substage condenser either directly, or via a mirror or prism. The condenser focuses or concentrates the available light into the plane of the object (Fig. 3.10). Generally, the more light at the specimen, the better the image resolution.

Condensers in microscopes are capable of vertical adjustment to allow for the varying heights or thickness of the slides and once the correct position has been established it should not be moved, as any alteration will change the light intensity and impair the resolution. Condensers are usually provided with adjustment screws. The screws allow centering of the light path which should be routinely checked before using the instrument. The diameter of the light beam can be controlled via the aperture diaphragm of the condenser.

Adjustment of the iris diaphragm will alter the size and volume of the cone of light focused on the object. If the diaphragm is closed too much, there is increased contrast and the image becomes refractile. Leaving the diaphragm wide open will cause the image to suffer from *glare* due to extraneous light interference. In both cases the resolution of the image is poor. The correct setting for the diaphragm is when the numerical aperture of the condenser is matched to the numerical aperture of the objective in use (Fig. 3.11) and the necessary adjustment should be made when changing from one objective to another. This is achieved by removing the eyepiece, viewing the substage iris diaphragm in the back focal plane of the objective, and closing it down to two-thirds of the field of view. With experience the correct setting can be estimated from the image quality.

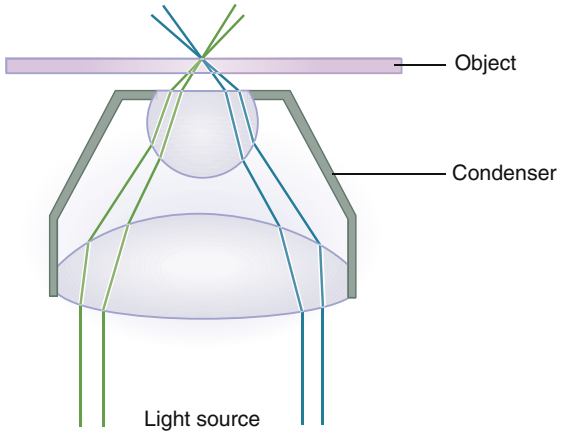

Fig. 3.10 The function of the condenser is to concentrate, or focus, the light rays at the plane of the object.

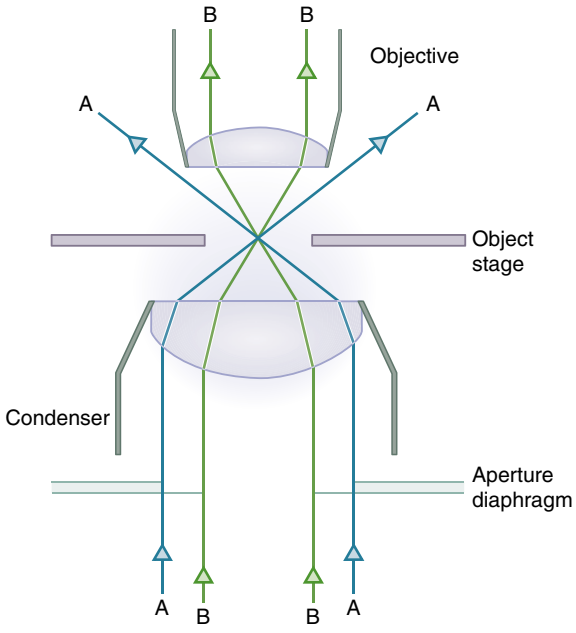

Fig. 3.11 Light rays A illustrate the 'glare' position resulting in extraneous light and poor resolution whereas B indicate the correct setting of the substage iris diaphragm.

The iris diaphragm should not be closed to reduce the intensity of the light, either use filters or alter the rheostat setting of the lamp transformer. In condensers fitted with a swing-out top lens, this is turned into the light path when the higher power objectives are in use. This focuses the light

into a field more suited to the smaller diameter of the objective front lens. Swing the top lens out of the path with the lower power objectives, or the field of view will only be illuminated at the center. Aplanatic or a highly corrected achromatic substage condenser should be used with an apochromatic or fluorite objective.

Object stage

This sits above the condenser and supports the glass slide. It is perpendicular to the optical path with an aperture for the light. The stage moves in two directions and Vernier scales enable the operator to return to an exact location on the specimen. The scale is also used for measuring the separation of various elements in the plane of section.

Objectives

The type and quality of the objective has the greatest influence on the performance of the microscope. There may be from 5 to 15 lens elements within the objective depending on the image ratio, type and quality (Fig. 3.12). The main task of the objective is to collect and unite the maximum amount of light possible from the object, forming a high-quality magnified real image above the lens unit.

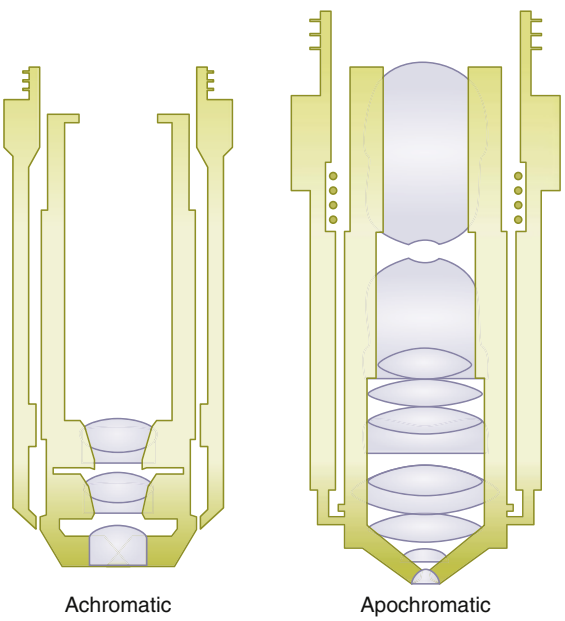

Fig. 3.12 Diagram of achromatic and apochromatic objectives.

The magnification power is usually inscribed on the side of the objective lens and is a reflection of the object: image ratio – most microscopes have 1:4, 1:10 and 1:40 as the basic minimum. The ability of an objective to resolve detail is indicated by its *numerical aperture* (NA), and not its magnifying power. This is expressed as a value calculated by a mathematical formula. The NA is the product of the refractive index of the medium between the lens and objective, and the sine of the angle between the optical axis of the lens and the outermost ray of light which can enter the front of the lens. The maximum NA attainable for a dry objective is 0.95, for water it is 1.3 and oil 1.5.

Resolution is the smallest distance between two dots or lines which can be seen as separate entities and is dependent on the wavelength of the light used and the NA of the lens. The *resolving power* of the objective is its ability to resolve the detail which can be measured, i.e. as the NA of an objective increases, the resolving power increases but working distance, flatness of field and focal length decrease.

Objectives are available in varying quality and types (Fig. 3.12.). *Achromatic objectives* are the most widely used for routine purposes; the more highly corrected *apochromats*, often incorporating fluorite glass, are used for more critical work, and *plan-apochromats*, which have a field of view which is almost perfectly flat, are recommended for photomicrography and cytology screening.

Objectives are designed for use with a coverglass protecting the object and a value giving the correct coverglass thickness, usually 0.17mm, is engraved on it. Apochromats between 40:1 and 63:1 require the coverslip thickness to be precise and some are mounted in a correction mount which can be adjusted to suit the thickness of the coverglass used.

The body tube and eyepiece

The image from the objective is formed in the body tube and magnified by the eyepiece, presenting the

eye with a virtual image which appears to be in the plane of the object. This image is observed at an optical distance 250mm from the eye. The body tube can be monocular, binocular or combined with photographic imaging tubes.

Using the microscope

The microscope should remain clean and well-maintained. Dust, finger prints and other materials, e.g. fragments of glass, whether on the substage, eyepiece or the gearing mechanism will impede the performance of any microscope. Only appropriate oils should be used on the stage to allow free movement of the object in two dimensions. The substage condenser and objectives should be freely mobile, but not loose. Only appropriate cleaning cloths for the lenses should be used, cleaning any oil immersion lens after each use. The light source should be appropriate for the microscope and centered if necessary by adjusting the condenser position. The filters used will depend on the type of microscopy. Coarse and fine focusing are achieved by moving the top tube and condenser lenses towards or away from the section on the slide being careful not to crush the object lens into the slide itself! When not in use the microscope should be covered.

Magnification

In a standard microscope with an optical tube length of 160mm, total magnification is the product of the magnification values of the objective and eyepiece. Using the combined magnifications of the objective (40x), tube length (1.25x), and eyepiece (10x), it is possible for a total magnification of 500x being seen by the observer.

Illumination

There have been varying approaches to maximize the illumination within the microscope. *Critical illumination* is used in simple equipment where the light source is focused by the substage condenser in the same plane as the object, but this produces uneven illumination. *Köhler illumination* is used for photography and more specialized microscopes where an image of the light source is focused by the lamp collector or field lens in the focal plane of the stage condenser on the aperture diaphragm. The image of the field or lamp diaphragm is focused in the object plane and the illumination is even (Fig. 3.13). The illumination must be centered with respect to the optical axis of the microscope to prevent poor resolution.

Dark field illumination

Occasionally it is preferable, or essential, that unstained sections or living cells are examined. These specimens and their components have refractive indices close to that of the medium in which they are suspended and are difficult to see by bright field techniques because of their lack of contrast. Dark field microscopy overcomes this by preventing direct light from entering the front of the objective and the only light gathered is that reflected, or diffracted by structures within the specimen (Fig. 3.14). This causes the specimen to appear as a bright image on a dark background, the contrast being reversed and increased. Dark field microscopy permits the detection of particles smaller than the optical resolution obtained in bright field microscopy due to the high contrast of the scattered light. Many small structures are more easily visualized by dark field techniques, although the resolution may be inferior to bright field microscopy. It is particularly useful for spirochetes, flagellates, cell suspensions, flow cell techniques, parasites and autoradiographic grain counting. Thin slides and coverglasses should be used and the preparation must be free of hairs, dirt, and bubbles.

Phase contrast microscopy

Unstained and living biological specimens have little contrast with their surrounding medium, although small differences of refractive index (RI) do exist in their structures. Phase contrast overcomes these

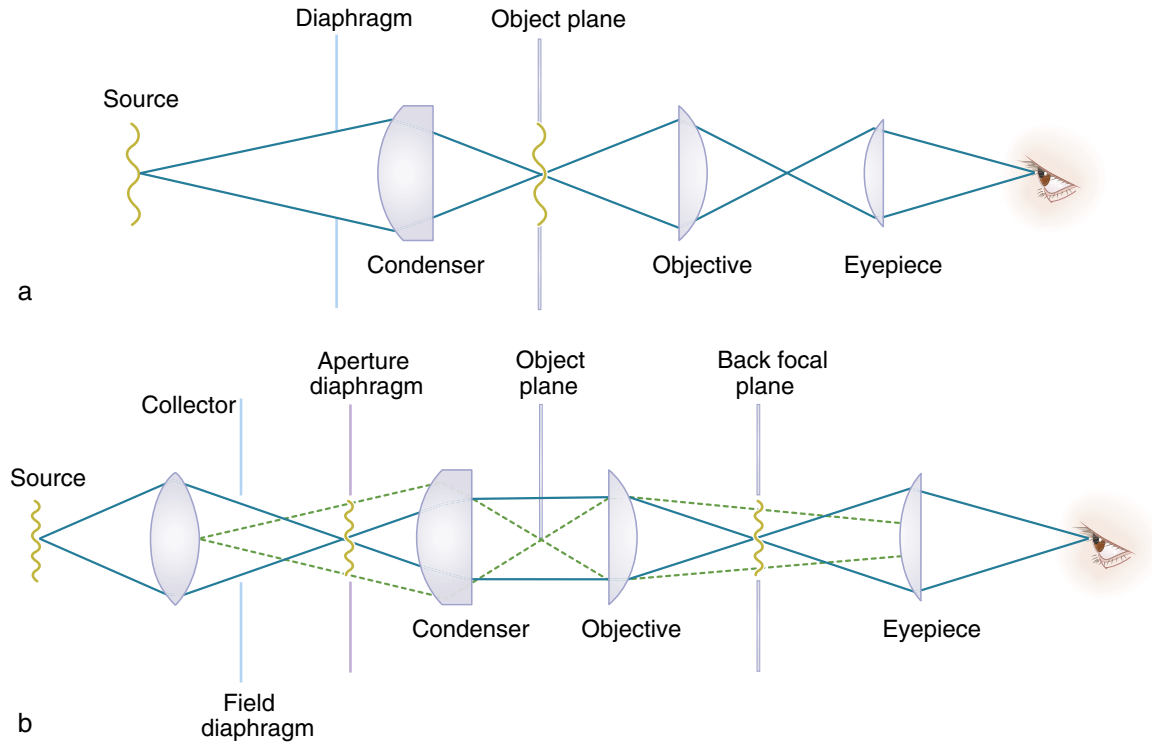

Fig. 3.13 **(a)** Critical illumination. **(b)** Köhler illumination.

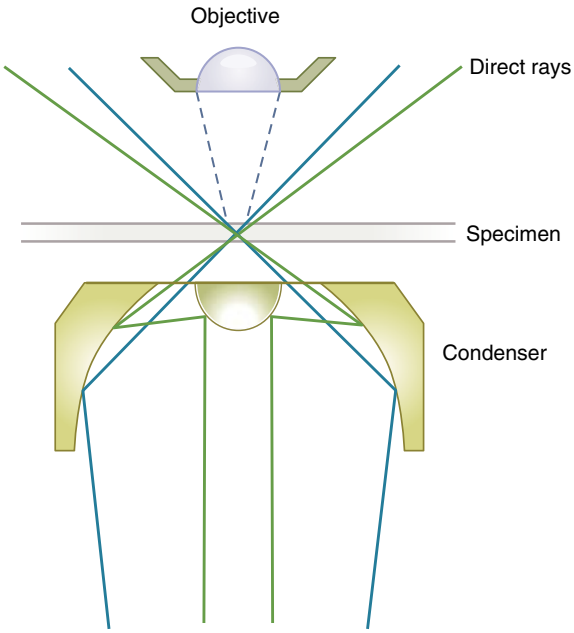

Fig. 3.14 In dark-field illumination no direct rays enter the objective: only scattered rays from the edges of structures within the specimen form the image (dashed lines).

problems by using controlled illumination with the full aperture of the condenser and therefore improving resolution. The higher the RI of a structure, the darker it will appear against a light background, i.e. with more contrast.

To achieve phase contrast, a microscope requires modification of the objectives and condenser, the specimen to retard light by between ⅛ and ¼ of the light wavelength (λ) and an intense Köhler illumination light.

Usually the microscope condenser carries a series of annular diaphragms made of opaque glass with a clear narrow ring which produce a controlled hollow cone of light. Each objective requires a different size of annulus, and an image of this is formed by the condenser in the back focal plane (BFP) of the objective as a bright ring of light. The objective is modified by a phase plate which is placed at its BFP (Fig. 3.15). A positive phase plate consists of a clear glass disc with a circular trough etched in it to half the depth of the disc. The light passing through the

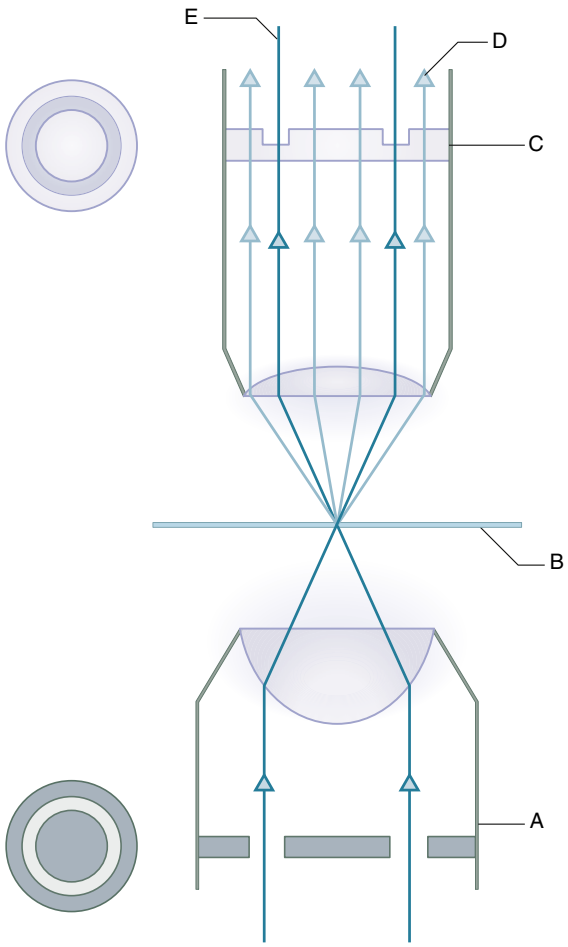

Fig. 3.15 The annulus, A, is at the focal plane of the condenser, B is the object plane and C the phase plate at the BFP. Light rays D are diffracted and retarded by the specimen with a total retardation of ½λ compared with the direct light, E, which is unaffected by the specimen.

trough has a phase difference of ¼ λ compared to the rest of the plate. The trough also contains a neutral density, light absorbing material to reduce the brightness of the direct rays, which would otherwise obscure the contrast obtained.

It is essential that the image of the bright annular ring from the condenser is centered and superimposed on the dull trough of the objective phase plate. This is achieved by using either a focusing telescope in place of the eyepiece, or a Bertrand lens (a small convergent lens), situated in the body tube of the microscope. Each combination of annulus and objective phase plate will require centering. When the hollow cone of direct light from the annulus enters the specimen, some of the cone will pass through unaltered, whilst some rays will be retarded or diffracted by approximately ¼ λ. The majority of the direct light will pass through the trough in the phase plate, whilst the diffracted rays pass through the thicker clear glass and are further retarded.

The total retardation of the diffracted rays is now ½ λ and interference will occur when they are recombined with the direct light. A contrasting image is achieved, revealing small details within unstained cells. This is a quick and efficient way of examining unstained paraffin wax, resin and frozen sections, as well as studying living cells and their behavior.

Interference microscopy

In phase contrast microscopy the specimen retards some of the light rays with respect to those passing through the surrounding medium. The resulting interference of these rays provides image contrast but with an artifact called the *phase halo*. In the interference microscope the retarded rays are entirely separated from the direct or reference rays, allowing improved image contrast and color graduation. Quantitative measuring of phase change (optical path difference), refractive index, dry mass of cells (optical weighing), and section thickness are also improved.

Polarized light microscopy

The use of polarized light in microscopy has many useful and diagnostic applications and it is recommended that cellular pathology laboratories have a simple system of polarizing microscopy as a minimum. Numerous crystals, natural and artificial fibrous structures, pigments, lipids, proteins, bone and amyloid deposits exhibit *birefringence*, i.e. the ability to produce plane polarized light. In histological material, birefringence is produced by asymmetric particles too small to be resolved even by the best lenses.

The polarizing microscope is a conventional microscope in which a Nicol prism or polarizing disc is interposed in the light path below the condenser. This polarizer converts all the light passing through the instrument into plane polarized light, i.e. light which vibrates in one optical plane only. A similar second prism, the analyzer, is placed within the barrel of the microscope above the objective lens. When the analyzer is rotated until its axis is perpendicular to that of the polarizer, no light can pass through the ocular lens resulting in a dark field effect. The field will remain black if an isotropic or singly refractive object is placed on the stage. A birefringent object however will appear bright upon the dark background.

Light entering a birefringent crystal, e.g. calcite, is split into two light paths, each determined by a different refractive index (RI) and each vibrating in only one direction, i.e. polarized but, at right angles to each other (Fig. 3.16). The higher the RI, the greater the retardation of the ray, so that each ray leaves the crystal at a different velocity. The high RI ray is called *slow* and the low RI ray is called *fast*. There is also a phase difference between the rays, and if they are recombined, interference occurs and various spectral colors are seen.

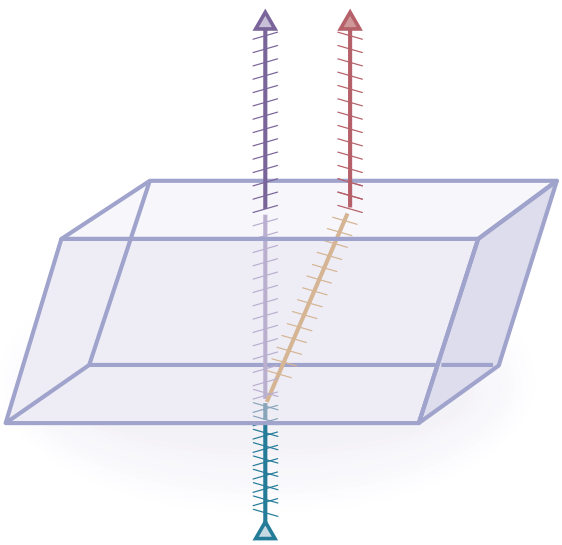

Fig. 3.16 A birefringent crystal such as calcite can split a ray of light into two light paths, each vibrating at right angles to the other.

Originally polarizers made from calcite and known as Nicol prisms, after their inventor, were cemented together with Canada balsam in such a way that the *slow* ray was reflected away from the optical path and into the mount of the prism, leaving only the polarized *fast* ray to pass through (Fig. 3.17).

The optic axis of a birefringent crystal is the direction in which light may pass unaltered. Substances through which light can pass in any direction and at the same velocity are *isotropic*, and are not able to produce polarized light. Some substances and crystals can produce plane polarized light by differential absorption and give rise to the phenomenon of *dichroism*.

The two phenomena detected in polarized light, birefringence and dichroism, are of value to the histologist. A dedicated polarizing microscope uses two polarizers (Fig. 3.18). One, always referred to as the *polarizer*, is placed beneath the substage condenser and held in a rotatable mount, this can be removed from the light path when not required. The other, the *analyzer*, is placed between the objective and the eyepiece.

When a birefringent substance is rotated between two crossed polarizers, the image appears and disappears alternately at each 45° of rotation, i.e. in a

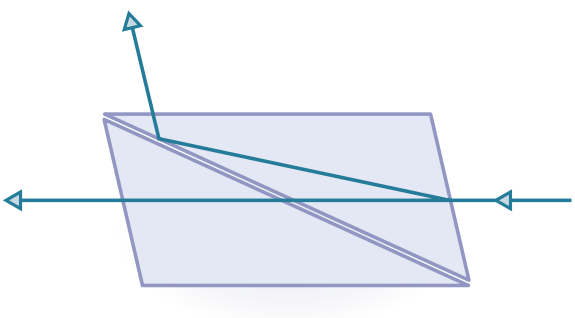

Fig. 3.17 A Nicol prism is constructed so that one part of the ray is allowed to pass whilst the other is directed away from the optical path and is lost.

complete revolution of 360° the image appears and is extinguished four times. When one of the planes of vibration of the object is in a parallel plane to the polarizer only one part ray can develop, and its further passage is blocked by the analyzer in the crossed position. However, at 45°, phase differences between the two rays can develop and are able to combine in the analyzer to form a visible image.

Some birefringent substances are also dichroic, being capable of emitting two colors. Only the polarizer is used and, if no rotating stage is available, the polarizer itself can be rotated. Changes in intensity and color are seen during rotation to 90° and back to its original color in the next 90° (Fig. 3.19). This is due to the differential absorption of light depending upon the vibration direction of the two rays in a birefringent substance.

Weak birefringence in biological specimens is enhanced by the addition of dyes, e.g. Congo Red for amyloid, or impregnating metals in an orderly linear alignment. Although only one polarizer is needed to detect the resulting dichroism, adding an analyzer can enhance the image.

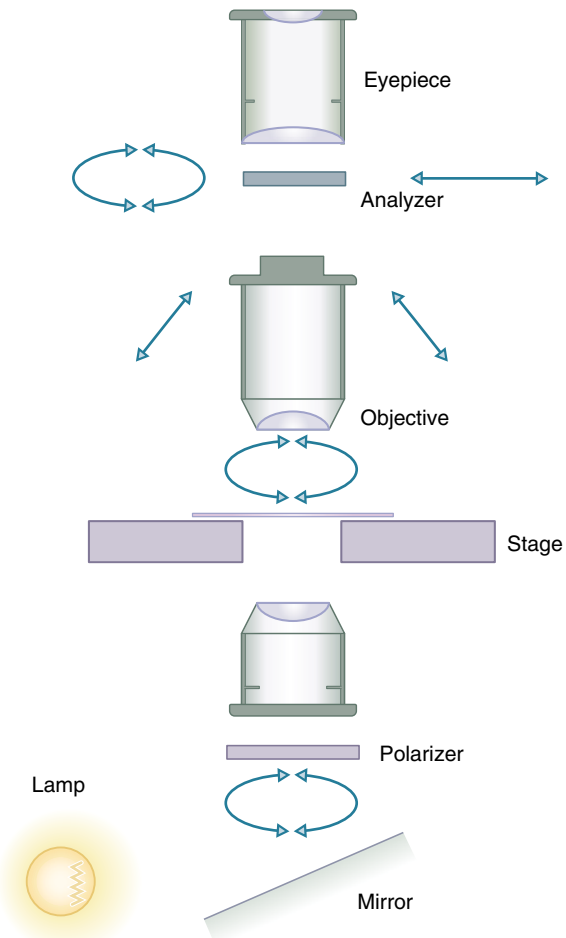

Fig. 3.18 A microscope equipped for polarized light.

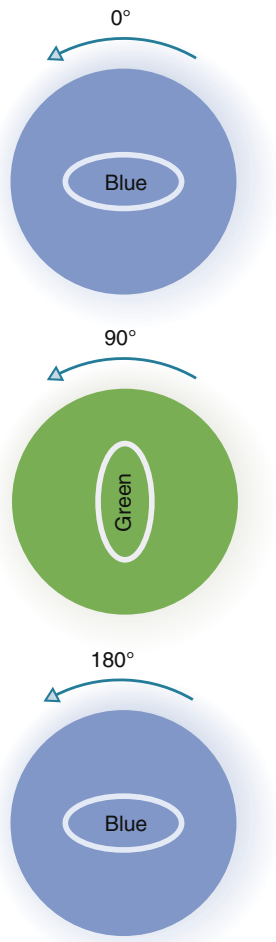

Fig. 3.19 When a dichroic substance is rotated in polarized light using the polarizer only, changes of color and intensity can be seen after rotating 90°. The original color returns after a further 90° rotation.

The *sign of birefringence* is diagnostically useful and is determined by the use of a compensator, i.e. a birefringent plate of known retardation, either above the specimen or below the polarizer at 45° to the direction of polarized light. If the *slow* ray (higher RI) is parallel to the length of the crystal or fiber, the birefringence is *positive*. If the *slow* ray is perpendicular to the long axis of the structure, the birefringence is *negative*. Rotating the compensator or the specimen until the *slow* direction of the compensator is parallel to the long axis of the crystal or fiber turns the field red and if the crystal is *blue* the birefringence is *positive*. If the crystal is *yellow*, the *slow* direction of the compensator is parallel to the *fast* direction of the crystal and the birefringence is *negative*. Quartz and collagen exhibit positive birefringence whilst Polaroid discs, calcite, urates and chromosomes are negatively birefringent.

Fluorescence microscopy

Fluorescence is the property of certain substances which when illuminated by light of a specific wavelength will re-emit this light at a longer wavelength. In fluorescence microscopy, the exciting radiation is usually in the ultraviolet wavelength, approximately 360nm or, the blue region, approximately 400nm, although longer wavelengths can be used with some modern dyes.

A substance which possesses a *fluorophore* will fluoresce naturally. This is known as *primary fluorescence* or *autofluorescence*. Ultraviolet excitation is required for optimum results with substances such as vitamin A, porphyrins and chlorophyll. Dyes, chemicals and certain antibiotics added to tissues produce *secondary fluorescence* of structures and are called *fluorochromes*. The majority of fluorochromes require only blue light excitation and this is the most common use of fluorescence in microscopy. *Induced fluorescence* is a term applied to substances such as catecholamines which, after treatment with formaldehyde vapor, are converted to fluorescent quinoline compounds.

Transmitted light fluorescence

All light sources emit a wide range of wavelengths, including the shorter ultraviolet and blue wavelengths of interest in fluorescence. Only a few sources emit sufficient short wave light for practical use and originally the most commonly used were high pressure mercury vapor or xenon gas lamps. Halogen filament lamps can produce enough light for some wavelength excitation in the blue and green range. The choice of a suitable source depends upon the type of work to be performed. Traditionally, mercury vapor burners were used for routine observation purposes, these operated on alternating current and their starting equipment was not expensive. However, they were toxic, required warming up and the bulb needed to cool before further use. These high pressure gas lamps have now been replaced by LED technology and the new LEDs used in fluorescence microscopes do not have these problems.

Preparations for fluorescence may contain other fluorescing material in addition to that in which one is interested. It is necessary to filter out all but the specific excitation wavelength, to avoid confusion between the important and the unimportant fluorescence within the sample being examined.

A variety of filters are available for this purpose. Dyed in the glass filters, e.g. UG 1 and BG 12 are broadband filters and transmit a wide range of wavelengths, the width of the range depending upon the composition and thickness of the filter. It is better to employ filters of a narrower band transmission which have their transmission peaks closer to the excitation maximum of the fluorochrome being used, e.g. FITC. Narrow band filters are often of the *interference* type, and are vacuum-coated layers of metals on a glass support. They have a mirror-like surface, and must be inserted in the beam with their reflective face towards the light source.

Barrier or suppression filters are placed before the eyepiece to prevent short wavelength light damaging the retina of the observer (Fig. 3.20). However, they must allow the fluorescing color to

pass, otherwise a negative result may be obtained. Barrier filters are colorless, through yellow to dark orange and are of specific wavelength transmission, e.g. a K.470 filter will block all wavelengths below 470nm.

Bright field condensers are able to illuminate the object using all the available energy, but they direct the rays beyond the object into the objective which is a potential hazard to the eyes of the observer. They can also set up disturbing autofluorescence in the objective itself. A dark field condenser is therefore used in most systems. This does not allow direct light into the objective and it is more likely to give a dark contrasting background to the fluorescence. Only about 10% of the available energy is used and this is dependent on the design of the condenser.

Simple achromatic objectives should be used with bright field illumination to prevent autofluorescence. However, with dark-ground illumination, the range of objectives is considerably widened, and more elaborate lenses with higher apertures and better light gathering power are possible.

Incident light fluorescence

Incident illumination, or lighting from above and through the objective down to the object (Fig. 3.21) has a number of advantages over using the transmitted route. In principle, the excitation beam, after passing the selection filters, is diverted through the objective, on to the preparation where fluorescence is stimulated. This fluorescence travels back to the observer by the normal route (Fig. 3.21a). Dichroic mirrors have been produced which divide and divert the beam and are therefore able to transmit light of some wavelengths and reflect other wavelengths (Fig. 3.21b). Selecting the appropriate mirror, the desired wavelength is reflected to the object and the remainder passes through to be lost. The visible fluorescent light is collected by the objective in the normal way, passes to the eyepiece and any excitation rays bouncing back from the slide and coverglass are reflected back along their original path to the source and prevented from reaching the observer. The objective in this system also acts as a condenser, so the illumination and objective numerical apertures are the same, optically correct, and in their most efficient condition. Fluorescence is stimulated on the observer's side of the preparation and is therefore more brilliant, not being masked by covering material or section thickness.

Any type of objective, including sophisticated phase contrast and interference contrast objectives, can be used for simultaneous transmitted illumination with normal tungsten lighting,

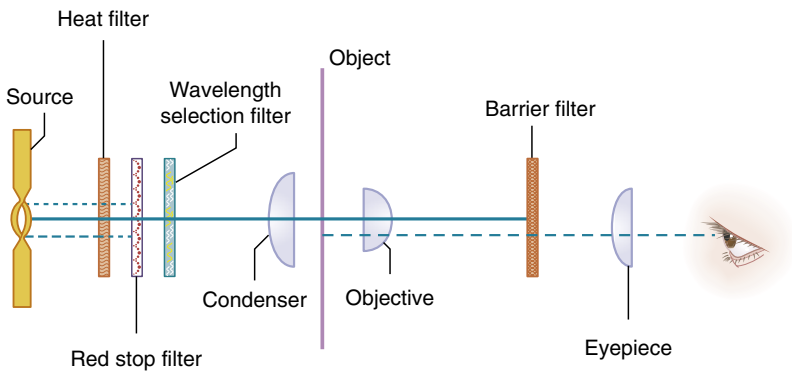

Fig. 3.20 Light path for transmitted fluorescence. Light of all wavelengths pass from the source through a heat-absorbing filter, into a second filter which removes red light, and then through a wavelength selection filter which allows only the desired excitation wavelength(s) to pass. On passing through the specimen, the objective collects both exciting and fluorescent wavelengths. The former is removed by a barrier filter to protect the eye of the observer.

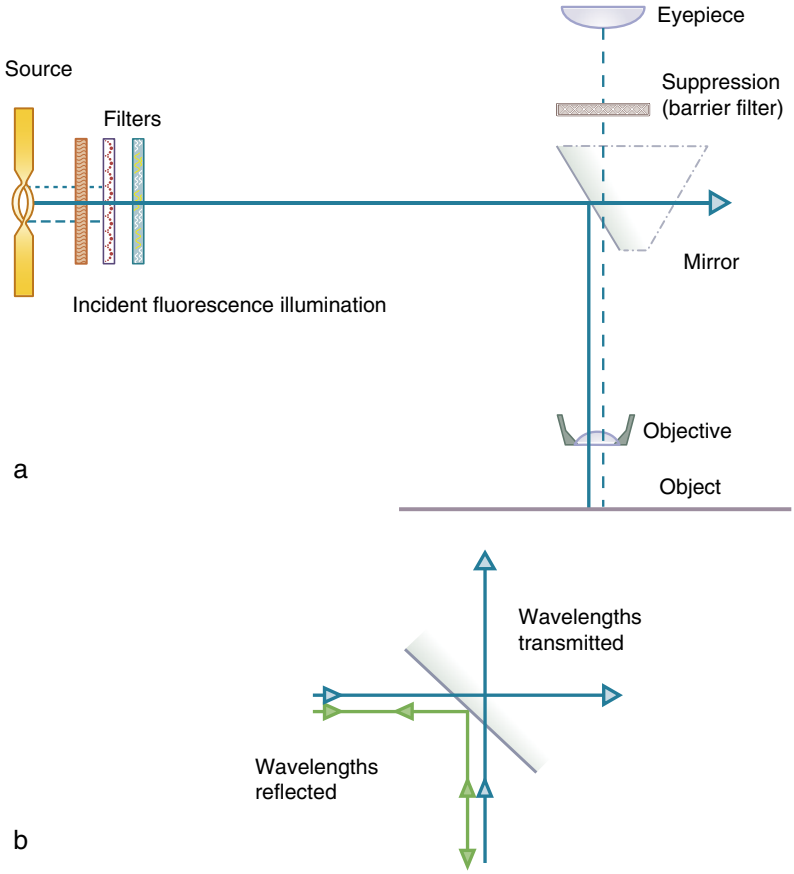

Fig. 3.21 **(a)** Diagram of an incident fluorescence microscope layout. **(b)** Effect of a dichroic mirror.

allowing the demonstration of both fluorescence and the morphology of the preparation. This is useful when normal stains cannot be used for fear of masking any fluorescent reactions. Brighter images are seen if dichroic mirrors are used as up to 90% of the excited energy can reach the preparation and 90% of the resultant visible light can be presented to the eye. Oil and water immersion objectives in low and high powers have been developed, they have higher numerical apertures and can gather more light, avoiding much of the lost stray light reflected from coverslips. The use of low magnification eyepieces improves fluorescence techniques.

The filters and light sources used in fluorescence microscopy in modern systems rely on digital image capture, and these images are monochromatic, i.e. black and white images. The highly colored fluorescence images which appear in publications are the result of pseudo coloring composite images.

The confocal microscope

Using conventional epifluorescence microscopes in fluorescence microscopy, the fluorochrome present in the field of view will be excited whether it is in, or out of focus. The out of focus fluorescence will reduce the contrast and resolution of the image. The confocal system uses a pinhole stop to observe the specimen, excluding the out of focus portion of the image. The axial resolution in the confocal system is greatly improved to 0.35mm in reflection,

with additional small but important gains in lateral resolution. This method therefore lends itself to optical sectioning. With modern computer technology and software, a series of optical sections can be recombined to create a 3D image of a cell or structure even when using multiple labeling techniques.

4 Fixation of tissues

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Introduction

This chapter discusses the basics of fixation, alongside the advantages and disadvantages of specific fixatives. It also provides some of the formulas for these fixatives currently used in pathology, histology and anatomy.

It is fair to say that the appropriate fixation of tissues for histological examination is central to all histology tests, as without this process all tissues would degrade and analysis would be useless. The last century has seen the development of a range of fixatives, with few recent modifications. The mechanisms and principles by which specific fixatives act fall into several broad groups. These include the covalent addition of reactive groups and cross-links, dehydration, the effects of acids, salt formation, and heat. Compound fixatives may function using several of these mechanisms.

When choosing a fixative there is a balance between the advantages and disadvantages which each fixative possesses. These include molecular changes or losses from 'fixed' tissues, swelling or shrinkage of tissues, variations in the quality of histochemical and immunohistochemical staining, the effect on biochemical analysis and the ability to maintain the structure of cellular organelles.

The major objective of fixation in pathology is to maintain clear and consistent morphological features (Eltoum et al., 2001a, 2001b; Grizzle et al., 2001). The development of specific fixatives has usually been empirical, although much of the understanding of the mechanisms of fixation has been based upon information obtained from leather tanning and vaccine production. In order to visualize the microanatomy of stained tissue sections, the original microscopic relationships between cells, cellular components (e.g. the cytoplasm and nuclei) and the extracellular material must be maintained with little disruption to the organization of the tissue. The local chemical composition of the tissue must also be maintained. Many tissue components are soluble in aqueous acid or other liquid environments and to reliably view the microanatomy and microenvironment of these tissues the soluble components must not be lost during fixation and tissue processing. Minimizing the loss of cellular components which include large proteins, small peptides, mRNA, DNA and lipids, prevents the destruction of macromolecular structures such as cytoplasmic membranes, smooth endoplasmic reticulum, rough endoplasmic reticulum, nuclear membranes, lysosomes and mitochondria. Each fixative, combined with the tissue processing protocol, maintains some molecular and macromolecular aspects of the tissue better than other fixative/processing combinations. If soluble components are lost from the cytoplasm of cells, the color of the cytoplasm on hematoxylin and eosin (H&E) staining will be reduced or modified and aspects of the appearance of the microanatomy of the tissue, e.g. mitochondria, will be lost or damaged. Similarly, immunohistochemical evaluations of structure and function may be reduced or lost.

Almost any method of fixation induces shrinkage or swelling, hardening of tissues and color variations in various histochemical stains (Sheehan & Hrapchak, 1980; Horobin, 1982; Fox et al., 1985; Carson, 1990; Kiernan, 1999; O'Leary & Mason, 2004). Various methods of fixation always produce some artifacts in the appearance of tissue on staining. However, for

diagnostic pathology it is important that such artifacts are consistent, predictable and understood.

The chosen fixative acts by minimizing the loss or enzymatic destruction of cellular and extracellular molecules, maintaining macromolecular structures and protecting tissues from destruction by microorganisms. This results in one view of a dynamically changing, viable tissue (Grizzle et al., 2001). The fixative should also prevent the subsequent breakdown of the tissue or molecular features by enzymatic activity and/or microorganisms during long term storage. These tissues removed from patients are an important resource which may at a later stage be subjected to further specialized tests, e.g. DNA-related or gene analysis.

A fixative not only interacts initially with the tissue in its aqueous environment but it also has ongoing reactivity with any unreacted fixative and the chemically altered tissues. Fixation interacts with all phases of processing and staining from dehydration to staining of tissue sections using histochemical, enzymatic or immunohistochemical stains (Eltoum et al., 2001b; Rait et al., 2004). It follows that any stained tissue section, produced after specific fixation combined with tissue processing, is a compromise of fixed tissue changes formed from the natural living tissue.

To date, a universal or ideal fixative has not been identified. Fixatives are therefore selected based on their ability to produce a final product needed to demonstrate a specific feature of a specific tissue (Grizzle et al., 2001). In diagnostic pathology, the fixative of choice for most pathologists has been 10% neutral buffered formalin (Grizzle et al., 2001).

An important constraint in using formaldehyde has been the loss of antigen immunorecognition due to this type of fixation combined with processing the tissue to paraffin wax (Eltoum et al., 2001a, 2001b). However, from a clinical perspective the advent of heat-induced epitope retrieval methods, instigated in the early 1990s, have overcome many of these limitations (Shi et al., 1991). Similarly, the analysis of mRNA and DNA from formalin-fixed, paraffin-embedded tissue has been problematic (Grizzle et al., 2001; Jewell et al., 2002; Steg et al., 2006; Lykidis et al., 2007). All widely used fixatives are therefore selected by compromise, with their positive aspects balancing against their less desirable features.

The most important characteristic of a fixative is to support high quality and consistent staining with H&E, both initially and after storage of the paraffin blocks for at least a decade, although new guidelines within the United Kingdom recommend that paraffin processed blocks are now kept for 30 years. The fixative must have the ability to prevent short and long term destruction of the micro-architecture of the tissue by stopping the activity of catabolic enzymes and hence autolysis, minimizing the diffusion of soluble molecules from their original locations. Another important characteristic of a good fixative, which helps maintain tissue and cellular integrity, is the fixation and inactivation of infectious agents.

It is also important to have good toxicological and flammability profiles which permit the safe use of the fixative (Grizzle & Fredenburgh, 2005). The advent of new biological methods, increased understanding of the human genome and the need to rapidly evaluate the biology of disease processes means that fixatives should also permit the recovery of macromolecules including proteins, mRNA, and DNA from fixed and paraffin-embedded tissues without extensive biochemical modifications.

Other important characteristics of an ideal fixative include being useful for a wide variety of tissue types, including fat, lymphoid and neural tissues. It should preserve small and large specimens and support histochemical, immunohistochemical, in situ hybridization and other specialized procedures. The fixative should penetrate and fix tissues rapidly, have a shelf life of at least one year and be compatible with modern automated tissue processors. It should be readily disposable or recyclable, support long term tissue storage to give excellent microtomy of paraffin blocks and should be cost effective (Dapson, 1993).

Types of fixation

Fixation of tissues can be accomplished by physical and/or chemical methods. Physical methods, e.g. heating, microwaving and freeze-drying are independent processes and not used commonly in the

routine practice of medical or veterinary pathology, anatomy and histology. The exception is the use of dry heat fixation of microorganisms prior to Gram staining. Most methods of fixation used in the processing of tissue for histopathological diagnoses rely on chemical fixation carried out by liquid fixatives. Reproducibility of the microscopic appearances of tissues after H&E staining is the prime requirement of the fixatives used for diagnostic pathology. Methods of fixation used in research protocols include the use of vapors and rarely, when fixation of a whole animal is needed, the perfusion of the animal's vascular system with a fixative (Eltoum et al., 2001a, 2001b).

Several chemicals, or their combinations, can act as good fixatives and accomplish many of the stated goals of fixation. Some fixatives add covalent reactive groups which may induce cross-links between proteins, individual protein moieties within nucleic acids and between nucleic acids and proteins (Horobin, 1982; Eltoum et al., 2001a, 2001b; Rait et al., 2004, 2005). The best examples of such 'cross-linking fixatives' are formaldehyde and glutaraldehyde.

Another approach to fixation is to use agents which remove free water from tissues and precipitate and coagulate the proteins. Examples of these dehydrants include ethanol, methanol and acetone. These agents denature proteins by breaking the hydrophobic bonds responsible for maintaining the tertiary structure of proteins. Other fixatives, e.g. acetic acid, trichloroacetic acid, mercuric chloride and zinc acetate act by denaturing proteins and nucleic acids through changes in pH or via salt formation.

Some fixatives are mixtures of reagents and are referred to as compound fixatives, e.g. alcoholic formalin fixes tissues in two ways: firstly, by adding covalent hydroxymethyl groups and cross-linking proteins and secondly, by coagulation and dehydration.

Physical methods of fixation

Heat fixation

This is the simplest form of fixation. Boiling or poaching an egg precipitates the proteins and, on cutting, the yolk and egg white can be identified separately. Each component is less soluble in water after heat fixation than the same component of a fresh egg. Picking up a frozen section on a warm microscope slide, both attaches the section to the slide and partially fixes it by heat and dehydration. Even though adequate morphology could be obtained by boiling tissue in normal saline, heat is primarily used to accelerate other forms of fixation as well as the other steps of tissue processing.

Microwave fixation

Microwave heating can reduce times for fixation of some gross specimens and histological sections from more than 12 hours to less than 20 minutes (Kok & Boon, 2003; Leong, 2005). Microwaving tissue in formalin results in the production of large amounts of dangerous, potentially explosive vapors. In the absence of a hood for extraction or a microwave processing system designed to handle these vapors, this may cause safety problems. Commercial glyoxal-based fixatives which do not form vapors when heated at 55°C have been introduced as an efficient method of microwave fixation.

Freeze-drying and freeze substitution

Freeze-drying is a useful technique for studying soluble materials and small molecules. Tissues are cut into thin blocks, immersed in liquid nitrogen and the water removed in a vacuum chamber at −40°C. The tissue can be post-fixed with formaldehyde vapor. In freeze substitution, specimens are immersed in fixatives, e.g. acetone or alcohol at −40°C, this slowly removes water through dissolution of ice crystals and the proteins are not denatured. Bringing the temperature gradually up to 4°C will complete the fixation process (Pearse, 1980). These methods of fixation are used primarily in the research environment and are rarely used in the clinical laboratory setting.

Chemical fixation

This utilizes organic or non-organic solutions to maintain adequate morphological preservation. Chemical fixatives can be considered as members of three major categories: coagulant, cross-linking, and compound (Baker, 1958).

Coagulant fixatives

Both organic and non-organic solutions may coagulate proteins making them insoluble. Cellular architecture in vivo is maintained primarily by lipoproteins and fibrous proteins such as collagen. Coagulating these proteins maintains tissue histomorphology at the light microscope level. Unfortunately, because coagulant fixatives result in cytoplasmic flocculation and poor preservation of mitochondria and secretory granules, these fixatives are not useful in ultrastructural analysis.

Dehydrant coagulant fixatives

The most commonly used in this group are alcohols (e.g. ethanol, methanol) and acetone. Methanol is closer to the structure of water than ethanol. Ethanol therefore competes more strongly than methanol in the interaction with hydrophobic areas of molecules and coagulant fixation begins at a concentration of 50–60% for ethanol but 80% or more for methanol (Lillie & Fullmer, 1976). The removal and replacement of free water from tissue by any of these agents has several potential effects on proteins within the tissue. Water molecules surround hydrophobic areas of proteins and, by repulsion, force hydrophobic chemical groups into closer contact with each other stabilizing hydrophobic bonding. By removing water, the opposite principle weakens hydrophobic bonding. Similarly, molecules of water participate in hydrogen bonding in hydrophilic areas of proteins, and therefore removal of water destabilizes this hydrogen bonding. Together, these changes act to disrupt the tertiary structure of proteins. Additionally, with the water removed the structure of the protein may become partially reversed, with hydrophobic groups moving to the outside surface of the protein. Once the tertiary structure of a soluble protein has been modified, the rate of reversal to a more ordered soluble state is slow and most proteins after coagulation remain insoluble even if returned to an aqueous environment.

Disruption of the tertiary structure of proteins (i.e. denaturation) changes their physical properties, potentially causing insolubility and the loss of function. Even though most proteins become less soluble in organic environments, up to 13% of protein may be lost, e.g. with acetone fixation (Horobin, 1982). Factors which influence the solubility of macromolecules include:

- Temperature, pressure, and pH.
- Ionic strength of the solute.
- The salting-in constant, which expresses the contribution of the electrostatic interactions.
- The salting-in and salting-out interactions.
- The types of denaturing reagents (Herskovits et al., 1970; Horobin, 1982; Papanikolau & Kokkinidis, 1997; Bhakuni, 1998).

Alcohol denatures protein differently depending on the choice and concentration of alcohol, the presence of organic and non-organic substances and the pH and temperature of fixation.

The protein denaturing effect of ethanol is > phenols > water and polyhydric alcohols > monocarboxylic acids > dicarboxylic acids (Bhakuni, 1998).

Other types of coagulant fixative

The charges on the ionizable side chains, e.g. $–NH_2 \rightarrow NH_3^+$ and $COO^- \rightarrow COOH$, are changed using acid coagulants such as picric and trichloroacetic acid by the disruption of electrostatic and hydrogen bonding. These acids may also insert a lipophilic anion into a hydrophilic region and disrupt the tertiary structures of proteins (Horobin, 1982). Acetic acid coagulates nucleic acids but does not fix or precipitate proteins. It is therefore added to other fixatives to prevent the loss of nucleic acids. Trichloroacetic acid (Cl_3CCOOH) can penetrate hydrophobic domains of proteins and the anion produced ($–C–COO^-$) reacts with charged amine groups. This interaction precipitates proteins and extracts nucleic acids. Picric acid or trinitrophenol dissolves slightly in water to form an acid solution (pH 2.0). In reactions it forms salts with basic groups of proteins causing the proteins to coagulate. If the solution is neutralized, the precipitated protein may re-dissolve. Picric acid fixation produces brighter staining, but the low pH solution may cause hydrolysis and the loss of nucleic acids.

Non-coagulant cross-linking fixatives

Several chemicals were selected as fixatives secondary to their potential actions of forming cross-links both within and between proteins and nucleic acids.

Cross-linking may not be a major mechanism with short times of fixation and therefore 'covalent additive fixatives' may be a better name for this group. Examples include formaldehyde, glutaraldehyde and other aldehydes, e.g. chloral hydrate and glyoxal, as well as metal salts, e.g. mercuric and zinc chloride, and other metallic compounds, e.g. osmium tetroxide. Aldehyde groups are chemically and biologically reactive and are responsible for many histochemical reactions, e.g. the argentaffin reaction (Papanikolau & Kokkinidis, 1997).

Formaldehyde fixation

Formaldehyde, as 10% neutral buffered formalin (NBF) is the most common fixative used in diagnostic pathology. Pure formaldehyde is a vapor which, when completely dissolved in water forms a solution containing 37–40% formaldehyde and this aqueous solution is 'formalin'. The usual '10% formalin' used in the fixation of tissues is a 10% solution of formalin, which contains approximately 4% weight to volume of formaldehyde.

The reactions of formaldehyde with macromolecules are numerous and complex. French and Edsall (1945) and Fraenkel-Conrat and his colleagues (1948a, 1948b, 1949) meticulously identified most of the reactions of formaldehyde with amino acids and proteins using simple chemistry. In an aqueous solution, formaldehyde forms methylene hydrate, a methylene glycol as the first step in fixation (Singer, 1962).

$$H_2C=O+H_2O \rightarrow HOCH_2OH$$

Methylene hydrate reacts with several side chains of proteins to form reactive hydroxymethyl side groups (–CH_2–OH). When the current relatively short fixation times are used with 10% neutral buffered formalin (hours to days), the formation of hydroxymethyl side chains is the primary and characteristic reaction and the formation of actual cross-links may be rare.

Formaldehyde also reacts with nuclear proteins and nucleic acids (Kok & Boon, 2003; Leong, 2005). It penetrates between nucleic acids and proteins and stabilizes the nucleic acid-protein shell, also modifying nucleotides by reacting with the free amino groups as it does with proteins. In naked and free DNA, the cross-linking reactions are believed to start at adenine-thymidine rich regions and cross-linking increases with rising temperatures (McGhee & von Hippel, 1975a, 1975b, 1977a, 1977b). Formaldehyde reacts with C=C and –SH bonds in unsaturated lipids but does not interact with carbohydrates (French & Edsall, 1945; Hayat, 1981).

The side chains of peptides or proteins which are most reactive with methylene hydrate have the highest affinity for formaldehyde; these include lysine, cysteine, histidine, arginine, tyrosine and the reactive hydroxyl groups of serine and threonine (Table 4.1) (Means & Feeney, 1995).

Gustavson (1956) reported that one of the most important cross-links in 'over-fixation', i.e. in tanning, is between lysine and the amide group of the protein backbone but again with the current shorter fixation times this is unlikely to occur (French & Edsall, 1945; Fraenkel-Conrat et al., 1945, 1947; Fraenkel-Conrat & Olcott, 1948a, 1948b; Fraenkel-Conrat & Mecham, 1949; Gustavson, 1956).

Reversibility of formaldehyde-macromolecular reactions

The reactive groups may combine with hydrogen groups or with each other to form methylene bridges. If the formalin is washed away, reactive groups may rapidly return to their original states, but any bridging which has already occurred may remain. Washing for 24 hours removes approximately half of the reactive groups and after 4 weeks up to 90% are removed (Helander, 1994). This suggests that actual cross-linking is a relatively slow process and, most 'fixation' with formaldehyde prior to tissue processing in diagnostic pathology stops with the formation of reactive hydroxymethyl groups.

During long term storage in formalin, the reactive groups may be oxidized to the more stable groups (e.g. acids –NH–COOH) which are not easily removed by washing in water or alcohol. Returning the specimen to water or alcohol following fixation therefore reduces the further fixation of the specimen because the reactive groups produced by the initial reaction with formalin may reverse and be removed. Although it was initially thought that cross-linking was the most important process in the fixation of tissue for biological uses (based on the limited number of cross-links over short periods of fixation), it is likely

Table 4.1 Action of major single or combination fixatives

Category of fixative	Dehydrants	Aldehyde cross-linkers	Combination mercuric chloride with formaldehyde or acetic acid	Osmium tetroxide	Picric acid plus formalin and acetic acid	Combination alcohols plus formalin
Examples of category	Ethanol Methanol Acetone	Formaldehyde Glutaraldehyde	Zenker's B5	Post-fixation after glutaraldehyde	Bouin's	Alcoholic formalin
Effect on proteins	Precipitates without chemical addition	Cross-linkers: adds active hydroxymethyl groups to amines, amides, some reactive alcohols, and sulfydryl groups; cross-links amine/amide or sulfydryl side chains of proteins	Additive plus coagulation	Additive cross-links; some extraction, some destruction	Additive and non-additive coagulant, some extraction	Additive plus precipitation
mRNA/DNA	Slight	Slowly cross-links; slightly extracts	Coagulation	Slight extraction	No action	Slight
Lipids	Extensive extraction	No action	No action	Made insoluble by cross-links with double bonds	No action	Extensive extraction
Carbohydrates	No action	None on pure carbohydrates; cross-linking of glycoproteins	No action	Slight oxidation	No action	No action
Quality of H&E staining	Satisfactory	Good	Good	Poor	Good	Good
Effect on ultrastructure (organelles)	Destroys ultrastructure, including mitochondria, proteins, coagulates	Preservation with NBF good, excellent with glutaraldehyde and adequate to good with Carson-Millonig's	Poor preservation	Used for visualization of membranes	Poor – tends to destroy membranes	Poor

Continued

Table 4.1 Action of major single or combination fixatives—cont'd

Category of fixative	Dehydrants	Aldehyde cross-linkers	Combination mercuric chloride with formaldehyde or acetic acid	Osmium tetroxide	Picric acid plus formalin and acetic acid	Combination alcohols plus formalin
Usual formulation	70–100% solution or in combination with other types of fixative	Formaldehyde (37%) – 10% V/V aqueous solution buffered with phosphates to pH 7.2–7.4. Glutaraldehyde – 2% buffered to pH 7.4	Mercuric chloride combined either with acetic acid plus dichromate or with formaldehyde plus acetate	1% solution buffered to pH 7.4	Aqueous picric acid, formalin, glacial acetic acid	10% formaldehyde (37%) with 90% ethanol
Important variables/ issues	Time, specimen thickness – should be used only for small or thin specimens	Time, temperature, pH, concentration/ specimen thickness	Toxic	Extremely toxic	Mitochondria and integrity of nuclear membrane destroyed; not appropriate for some stains	Time, specimen dimensions. Note good fixative for renal tissues
Special uses	Preserves small non-lipid molecules such as glycogen; preserves enzymatic activity	General all-round fixative; best for ultrastructure if used with osmium tetroxide post-fixation	Excellent for hematopoietic tissues	Ultrastructural visualization of membranes; lipids on frozen sections	Mordant for connective tissue stains (trichrome)	Good general fixative; good for specific immunohistochemical reactions and good to detect lymph nodes in fatty tissue; removes fats from tissue

that the formation of these hydroxymethyl groups actually denatures macromolecules and renders them insoluble. As these washing experiments have not been reproduced, the actual mechanisms and their importance in fixation by formaldehyde remain uncertain.

Over-fixation of tissue may also be partially corrected by soaking the tissue in concentrated ammonia plus 20% chloral hydrate (Lhotka & Ferreira, 1949). Fraenkel-Conrat and his colleagues noted that the addition and condensation reactions of formaldehyde with amino acids and proteins were unstable and could be reversed easily by dilution or dialysis (Fraenkel-Conrat et al., 1945, 1947; Fraenkel-Conrat & Olcott, 1948a, 1948b; Fraenkel-Conrat & Mecham, 1949).

The principal type of cross-link in short term fixation is thought to be between the hydroxymethyl group on a lysine side chain and arginine (through secondary amino groups), asparagine and glutamine (through secondary amide groups) or tyrosine (through hydroxyl groups) (Tome et al., 1990). For example, a lysine hydroxymethyl amine group can react with an arginine group to form a lysine–CH_2–arginine cross-link. Similarly, a tyrosine hydroxymethyl amine group can bind with a cysteine group to form a tyrosine–CH_2–cysteine cross-link. Each of these cross-links between macromolecules has a different degree of stability which can be modified by the temperature, pH and the type of environment surrounding and permeating the tissue (Eltoum et al., 2001b). The time to saturation of human and animal tissues with active groups by formalin is approximately 24 hours, but cross-linking may continue for many weeks (Helander, 1994).

When formaldehyde dissolves in an unbuffered aqueous solution, it forms an acid solution (pH 5.0–5.5) because 5–10% of commercially available formaldehyde is formic acid. Acid formalin may react more slowly with proteins than NBF because the amine groups become charged, e.g. $–N^+H_3$. In solution, this requires a much lower pH than 5.5. However, the requirement for a lower pH to produce $–N^+H_3$ groups may not be equivalent to that required in peptides. Acid formalin also preserves immunorecognition better than NBF (Arnold et al., 1996). Indeed, the success of early immunocytochemistry methods to demonstrate immunoglobulins in paraffin-processed tissue sections probably relied on the fixation of the tissues in acid formalin (Taylor & Burns, 1974). The disadvantage of using acid formalin for fixation is the formation of a brown/black pigment with degraded hemoglobin. This heme-related pigment which forms in tissue, is not a problem unless patients have a blood abnormality, e.g. sickle cell disease or malaria.

Formaldehyde primarily preserves peptide-protein bonds and the general structure of cellular organelles. It can interact with nucleic acids but has little effect on carbohydrates and preserves lipids if the solutions contain calcium (Bayliss High & Lake, 1996).

Glutaraldehyde fixation

Less is known about the biological reactions and effects of glutaraldehyde compared to formaldehyde as it has not been used as widely in biological applications. Glutaraldehyde is a bifunctional aldehyde which probably combines with the same reactive groups as formaldehyde. In aqueous solutions glutaraldehyde polymerizes forming cyclic and oligomeric compounds (Hopwood, 1985); it is also oxidized to glutaric acid. It requires storage at 4°C and a pH of approximately 5 for stability (Hopwood, 1969).

Unlike formaldehyde, glutaraldehyde has an aldehyde group at both ends of the molecule. Following each reaction of the first group, an unreacted aldehyde group may be introduced into the protein and these groups can act to further cross-link the protein. Alternatively, the aldehyde groups may react with a wide range of other histochemical targets which include antibodies, enzymes or proteins. The reaction of glutaraldehyde with an isolated protein such as bovine serum albumin, is fastest at pH 6–7 and results in more cross-linking than formaldehyde (Habeeb, 1966; Hopwood, 1969). Cross-linking is irreversible and withstands acids, urea, semicarbazide and heat (Hayat, 1981). Similar to formaldehyde, reactions with lysine are the most important for forming cross-links.

Extensive cross-linking by glutaraldehyde results in better preservation of the ultrastructure, but this method of fixation negatively affects immunohistochemical methods and slows the penetration by the fixative. Any tissue fixed in glutaraldehyde must be small (0.5 mm maximum) and, unless the aldehyde groups are blocked, increased background staining will result (Grizzle, 1996a). Glutaraldehyde does not react with carbohydrates or lipids unless they

contain free amino groups which are found in some phospholipids (Hayat, 1981). At room temperature glutaraldehyde does not cross-link nucleic acids in the absence of nucleohistones, but it may react with nucleic acids at or above 45°C (Hayat, 1981).

Osmium tetroxide fixation

Osmium tetroxide (OsO_4) is a toxic solid which is soluble in water as well as non-polar solvents. It can react with hydrophilic and hydrophobic sites including the side chains of proteins where it potentially can cause cross-linking (Hopwood et al., 1990). The reactive sites include sulfydryl, disulfide, phenolic, hydroxyl, carboxyl, amide and heterocyclic groups. Osmium tetroxide is known to interact with nucleic acids, specifically the 2,3-glycol moiety in terminal ribose groups and the 5,6 double bonds of thymine residues. Nuclei fixed in OsO_4 and dehydrated with alcohol may show prominent clumping of DNA. This unacceptable artifact can be prevented by pre-fixation with potassium permanganate ($KMnO_4$), post-fixation with uranyl acetate or by adding calcium ions and tryptophan during fixation (Hayat, 1981). The reaction of OsO_4 with carbohydrates is variable (Hayat, 1981). Large proportions of proteins and carbohydrates are lost from tissues during osmium fixation. This may be due to the superficial penetration of OsO_4 (<1 mm) into tissues, or its slow rate of reaction. In electron microscopy this loss is minimized by initial fixation of tissue in glutaraldehyde.

The best characterized reaction of osmium is its reaction with unsaturated bonds within lipids and phospholipids. Osmium, in this reaction, alters from the +8 valence state to the +6 valence state, which is colorless. If two unsaturated bonds are close together there may be cross-linking by OsO_4. Although the complex is colorless at this point, the typical black staining of membranes expected from fixation with osmium requires the production of osmium dioxide ($OsO_2 \cdot 2H_2O$). Osmium dioxide is black, electron dense and insoluble in aqueous solution; it precipitates as the above unstable compounds break down and becomes deposited on cellular membranes. The breakdown of osmium +6 valence complexes to osmium dioxide (+4 valence state) is facilitated by a reaction with solutions of ethanol.

In addition to its use as a secondary fixative for electron microscope examinations, OsO_4 can be used to stain lipids in frozen sections. Osmium tetroxide fixation causes tissue swelling which is reversed during the dehydration steps. Swelling can also be minimized by adding calcium or sodium chloride to osmium-containing fixatives (Hayat, 1981).

Cross-linking fixatives for electron microscopy

Cell organelles, e.g. cytoplasmic and nuclear membranes, mitochondria, membrane-bound secretory granules, smooth and rough endoplasmic reticula, need to be preserved carefully for electron microscopy. The lipids in these structures are extracted by many dehydrating fixatives, e.g. alcohols, and for ultrastructural examination it is therefore important to use a fixative which does not remove lipids. Strong cross-linking fixatives are preferred, e.g. glutaraldehyde, a combination of glutaraldehyde and formaldehyde or Carson's modified Millonig's, followed by post-fixation in an agent such as OsO_4 which further stabilizes and emphasizes membranes.

Mercuric chloride fixatives

Historically, mercuric chloride was favored for its ability to enhance the staining properties of tissues, particularly with the trichrome stains. However, it is now rarely used in the clinical laboratory due to the health and safety issues of mercury-containing fixatives and, the reduced reliance on 'special stains'. A further major disadvantage of mercuric chloride fixation is the formation of intense black precipitates of mercuric pigment in the tissue which gives them inferior value for immunohistochemical and molecular studies. In recently fixed tissues, these precipitates can be removed by a Lugol's iodine step in the staining procedure, followed by bleaching of the section in sodium hypochlorite solution (Hypo). However, this is not effective on mercuric chloride fixed tissues which have been stored for a number of years as paraffin blocks. In these tissues, retrospective analysis by immunohistochemistry and molecular techniques is unreliable due to the formation of much larger aggregates of mercuric pigment which cannot be removed by Lugol's iodine.

The chemistry of fixation using mercuric chloride is not well understood but it is known that mercuric chloride reacts with ammonium salts, amines, amides, amino acids and sulfydryl groups to harden tissues. It is especially reactive with cysteine, forming a dimercaptide (Hopwood, 2002) which acidifies the solution:

$$\text{Sulfydryl} - 2\,(\text{R} - \text{S} - \text{H}) + \text{HgCl}_2 \rightleftharpoons (\text{R} - \text{S})_2 - \text{Hg} + 2\text{H}^+ + 2\text{Cl}^-$$

If only one cysteine is present, a reactive group of R–S–Hg–Cl is likely.

Mercury-based fixatives are toxic and all should be handled with care. They should not be allowed to come into contact with metal, and should be dissolved in distilled water to prevent the precipitation of mercury salts. Mercury-containing chemicals also pose an environmental disposal problem.

These fixatives penetrate slowly, so specimens must be thin, and mercury and acid formaldehyde hematein pigments may deposit in tissue after fixation. Mercury fixatives (Hopwood, 1973) are no longer used routinely, except, e.g. B5, by some laboratories for fixing hematopoietic tissues. A potential replacement for mercuric chloride is zinc sulfate. Special formulations of zinc sulfate in formaldehyde replacing mercuric chloride in B5 may give better nuclear detail than formaldehyde alone and improve tissue penetration (Carson, 1990).

Special fixatives

Dichromate and chromic acid fixation

Chromium trioxide dissolves in water to produce an acidic solution of chromic acid with a pH of 0.85 and this is a powerful oxidizing agent which produces aldehyde from the 1, 2-diglycol residues of polysaccharides. These aldehydes can react with histochemical stains, e.g. PAS and argentaffin/argyrophil and should increase the background of immunohistochemical staining (Grizzle, 1996a).

Actual chromic salts, i.e. chromium ions in +3 valence state, may destroy animal tissues (Kiernan, 1999), but chromium ions in their +6 state coagulate proteins and nucleic acids. The fixation and hardening reactions are not fully understood, but probably involve the oxidation of proteins which varies in strength depending upon the pH of the fixative, and the interaction of the reduced chromate ions directly in cross-linking proteins (Pearse & Stoward, 1980). Chromium ions specifically interact with the carboxyl and hydroxyl side chains of proteins and chromic acid interacts with disulfide bridges and attacks lipophilic residues such as tyrosine and methionine (Horobin, 1982). Fixatives containing chromate at a pH of 3.5–5.0 make proteins insoluble without coagulation. Chromate is reported to make unsaturated but not saturated lipids insoluble upon prolonged (>48 hours) fixation and hence mitochondria are well preserved by these fixatives.

Dichromate-containing fixatives have primarily been used to prepare neuroendocrine tissues for staining, especially normal adrenal medulla and its related tumors, e.g. pheochromocytomas. However, reliance on the chromaffin reaction used to identify chromaffin granules following dichromate fixation is now being replaced by immunohistochemistry with a range of neuroendocrine markers including chromagranin A and synaptophysin (Grizzle, 1996a, 1996b).

Fixatives for DNA, RNA and protein analysis

Lykidis et al. (2007) conducted a comprehensive analysis of 25 fixative compounds, many reputed to provide improved preservation of DNA, RNA and proteins in tissues for immunocytochemical analysis, whilst at the same time ensuring optimal morphological preservation. These compounds included the commercially available HEPES-glutamic acid buffer mediated Organic Solvent Protection Effect (HOPE) fixative, the reversible cross-linker dithiobis[succinimidyl propionate] (DSP) for immunocytochemistry and expression profiling, and zinc-based fixatives. They concluded that a novel zinc formation, Z7, containing zinc trifluoroacetate, zinc chloride and calcium acetate was significantly better than the standard zinc-based fixative, Z2 and NBF for DNA, RNA and antigen preservation. DNA and RNA fragments up to 2.4kb and 361bp in length respectively, were detected by a polymerase chain reaction (PCR), reverse transcriptase PCR and real-time PCR in the Z7 fixed tissues, also allowing protein analysis using 2D electrophoresis. Nucleic acids and protein were found to be stable over a period of 6–14 months. The fixative is also less toxic than formaldehyde formulations. Whilst this Z7 fixative has shown great promise, it should be borne in mind that fixation in NBF will also allow the extraction of similarly sized

fragments of DNA and RNA for analysis by PCR-based technologies within the same time frame.

Metallic ions as a fixative supplement

Several metallic ions have been used as aids in fixation, including Hg^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, $[UO_2]^{2+}$, $[PtCl_6]^{2+}$ and Zn^{2+}. Mercury, lead and zinc are used most commonly in current fixatives, e.g. zinc-containing formaldehyde is suggested to be a better fixative for immunohistochemistry than formaldehyde alone. This does however depend upon the pH of the formaldehyde, as well as the zinc formaldehyde (Arnold et al., 1996; Eltoum et al., 2001a).

Compound fixatives

Pathologists use formaldehyde-based fixatives to ensure reproducible histomorphometric patterns. Other agents may be added to formaldehyde to produce specific effects which are not possible with formaldehyde alone. The dehydrant ethanol, for example, can be added to formaldehyde to produce alcoholic formalin. This combination preserves molecules such as glycogen and results in less shrinkage and hardening than pure dehydrants.

Compound fixatives are useful for specific tissues, e.g. alcoholic formalin for fixation of some fatty tissues, such as breast, in which the preservation of the lipid is not important. Additionally, the fixation of gross specimens in alcoholic formalin may aid the identification of lymph nodes embedded in fat (see also page 55). Some combined fixatives, including alcoholic formalin, are good at preserving antigen immunorecognition, but non-specific staining or background staining in immunohistochemical procedures can be increased. Unreacted aldehyde groups in glutaraldehyde-formaldehyde fixation, for example, may increase background staining and alcoholic formalin may cause non-specific staining of myelinated nerves (Grizzle et al., 1995, 1997, 1998a, 1998b; Arnold et al., 1996; Grizzle, 1996b).

Factors affecting the quality of fixation

Buffers and pH

The effect of pH on fixation with formaldehyde may be profound depending upon the applications to which the tissues will be exposed. In a strongly acidic environment, the primary amine target groups ($–NH_2$) attract hydrogen ions and become unreactive ($–NH^+_3$) to the hydrated formaldehyde (methylene hydrate or methylene glycol), and carboxyl groups ($–COO^–$) lose their charges (–COOH). This may affect the structure of proteins. Similarly, the hydroxyl groups of alcohols (–OH) including serine and threonine may become less reactive in a strongly acidic environment. The extent of formation of reactive hydroxymethyl groups and cross-linking is reduced in unbuffered 4% formaldehyde (Means & Feeney, 1995) which is slightly acidic (French & Edsall, 1945), because the major methylene cross-links are between lysine and the free amino group on the side chains.

The decrease in the effectiveness of formaldehyde fixation and hence cross-linking in the slightly acid environment has led some authors to suggest that unbuffered formalin is a better fixative than NBF for the immunorecognition of many antigens (Arnold et al., 1996; Eltoum et al., 2001b). This aided the detection of antigens prior to the advent of heat-induced epitope retrieval methods in immunocytochemistry. However, minimal delay in effectively fixing labile antigens, such as the estrogen receptor, is vital in the immunohistochemical testing for a range of clinically important prognostic and predictive biomarkers. Whilst formaldehyde fixation remains the recommended method for optimal preservation of morphological features, proteins and nucleic acids in a clinical environment, the most reliable way of achieving optimal formalin fixation is through its buffering at pH 7.2–7.4, i.e. NBF.

At the acidic pH of unbuffered formaldehyde, hemoglobin metabolic products are chemically modified to form a brown-black, insoluble, crystalline, birefringent pigment. This pigment forms at a pH less than 5.7 and the extent of its formation increases in the pH range 3.0 to 5.0. Formalin pigment is easily recognized and should not affect diagnoses except in patients with large amounts of hemoglobin breakdown products secondary to hematopoietic diseases. The pigment is removed easily with an alcoholic solution of picric acid. Using NBF avoids the formation of formalin pigment and it is used as the preferred formaldehyde-based fixative.

Acetic and other acids work mainly through lowering pH and disrupting the tertiary structure of proteins. Buffers are used to maintain the optimum pH. The choice of a specific buffer depends on the type of fixative and analyte. Commonly used buffers are phosphate, cacodylate, bicarbonate, Tris and acetate. It is necessary to use low salt, buffered formalin in the new complex tissue processors in order to keep the machine 'clean' and reduce problems in its operation.

Duration of fixation and the size of specimens

The factors which govern diffusion of a fixative into tissue were investigated by Medawar (1941). He found that the depth in mm, d, reached by a fixative is directly proportional to the square root of the duration of fixation in hours, t, and expressed this relation as:

$$d = k\sqrt{t}$$

The constant, k, is the coefficient of diffusability which is specific to each fixative. Examples are 0.79 for 10% formaldehyde, 1.0 for 100% ethanol and 1.33 for 3% potassium dichromate (Hopwood, 1969). Thus, the time of fixation is approximately equal to the square of the depth to which the fixative must penetrate and most fixatives, such as NBF will penetrate tissue to the depth of approximately 1 mm in one hour, e.g. for a 10 mm sphere, the fixative will not penetrate to the center until $(5)^2$, 25 hours of fixation. It is important to note that the components of a compound fixative will penetrate the tissue at different rates so it is better if these fixatives are used with thin specimens.

Gross specimens should not rest on the bottom of a container of fixative, they should be separated from the bottom by wadded fixative-soaked paper or cloth allowing penetration of the fixative in all directions. Additionally, unfixed gross specimens which are to be cut and stored in fixative prior to processing, should not be thicker than 5 mm. When surgical specimens are to be processed to paraffin blocks the time of penetration by the fixative is more critical. Specific issues related to the processing of tissues have been reviewed by Grizzle et al. (2001) and Jones et al. (2001).

Fixation proceeds slowly – the period between the formation of reactive hydroxymethyl groups and the formation of a significant number of cross-links is unknown. Ninety percent of reactive groups can be removed by 4 weeks of washing (Helander, 1994), confirming that cross-linking is not a rapid process and may require weeks for the completion of potential bonds.

Proteins inactivate fixatives, especially in blood or bloody fluids, so these gross specimens should be washed with saline prior to being put into fixative. The fixative volume should be at least 10 times the volume of the tissue specimen for optimal, rapid fixation. Currently in some laboratories, thin specimens may be fixed in NBF for only 5–6 hours, including the short time of fixation in tissue processors. The extent of the formation of cross-links during such rapid NBF fixation is uncertain. Consequently, the formation of hydroxymethyl groups may predominate as opposed to the more resilient cross-linking. It has been suggested that rapid fixation is acceptable as long as histochemical staining remains adequate, and that immunohistochemistry and other molecular techniques are probably enhanced by shorter times of fixation using an aldehyde-based fixative, e.g. formaldehyde. However, studies investigating the time taken to adequately fix clinical cases of breast cancer tissue for subsequent immunohistochemical detection of estrogen receptors illustrate that this practice can be detrimental to the optimal preservation of important antigens, and should be avoided. Goldstein et al. (2003) found that 6–8 hours was the minimum time required to adequately fix breast tissue for immunohistochemical testing of estrogen receptors, regardless of the size and type of the specimen. Consequently, the guidelines for estrogen receptor and progesterone receptor testing produced by the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) recommend this minimal fixation time in neutral buffered formalin for all clinical breast cancer specimens (Hammond et al., 2010)

Temperature of fixation

The diffusion of molecules increases with rising temperature due to their more rapid movement and vibration, i.e. the rate of penetration of a tissue by formaldehyde is faster at higher temperatures. Microwaves therefore have been used to speed formaldehyde fixation by both increasing the temperature and molecular movements. Increased vapor levels however, are a safety problem (Grizzle & Fredenburgh, 2001, 2005). Most chemical reactions occur more rapidly at higher temperatures and therefore formaldehyde reacts more rapidly with proteins in these conditions (Hopwood, 1985). Closed tissue processors have their processing retort directly above the paraffin holding stations which are held at 60–65°C, making the retort slightly warmer than room temperature.

Concentration of fixative

Effectiveness and solubility primarily determine the appropriate concentration of fixatives. Concentrations of formalin above 10% tend to cause increased hardening and shrinkage (Fox et al., 1985). Additionally at higher concentrations formalin is present in its polymeric form which can be deposited as a white precipitate, as opposed to its monomeric form $HO(H_2CO)H$, which at 4% is more soluble (Baker, 1958). Ethanol concentrations below 70% do not remove free water from tissues efficiently.

Osmolality of fixatives and ionic composition

The osmolality of the buffer and fixative is important – hypertonic and hypotonic solutions lead to shrinkage and swelling respectively. The best morphological results are obtained with solutions which are slightly hypertonic (400–450 mOsm), although the osmolality for 10% NBF is about 1500 mOsm. Similarly, various ions (Na^+, K^+, Ca^{2+} and Mg^{2+}) can affect cell shape and structure regardless of the osmotic effect. The ionic composition of fluids should be as isotonic as possible to the tissues being fixed.

Additives

The addition of electrolytes and non-electrolytes to fixatives may improve the morphology of the fixed tissue. These additives include calcium chloride, potassium thiocyanate, ammonium sulfate and potassium dihydrogen phosphate. The electrolytes may react either directly with proteins causing denaturation, or independently with the fixatives and cellular constituents (Hayat, 1981). The choice of electrolytes to be added to fixatives used on a tissue processor may vary. Fixatives buffered with electrolytes such as phosphates may cause problems with some processors due to the precipitation of the salts. The addition of non-electrolyte substances such as sucrose, dextran and detergent has also been reported to improve fixation (Hayat, 1981).

Selecting or avoiding specific fixatives

The choice of a fixative is a compromise, balancing their beneficial and detrimental effects. Kiernan (1999) originally produced a table of the actions of fixatives which was later modified and published by Eltoum et al. (2001b), and Table 4.1 is a further modification of the latter.

The main problem with fixatives used in histological staining is the loss by solution or extraction of molecules which are the targets for specific histochemical methods. Typically, some molecules are soluble in aqueous fixatives, e.g. glycogen, whilst others are soluble in organic-based fixatives, e.g. lipids. Some fixatives may chemically modify the targets of histochemical staining and affect the quality of special stains, e.g. glutaraldehyde with silver stains. This includes the modification of staining secondary to changes in pH induced by fixation. A good discussion of the effects of fixation on histochemistry is by Sheehan and Hrapchak (1980).

The table of Sheehan and Hrapchak (1980) modified by Eltoum et al. (2001b) has been changed so that harmful methods of fixation can be identified rapidly. Table 4.2 is a further modification of this.

Fixation for selected individual tissues

Eyes

The globe must be firmly fixed in order to cut good sections for embedding. Eyes may be fixed in NBF,

Table 4.2 Stains and fixatives

Target of stain	Name or type of stain	Preferred fixative	Fixative best avoided
Amebas	Best's carmine	Alcohol or alcoholic formalin	Aqueous fixative
Cholesterol and cholesterol esters	Schultz's method Digitonin	10% NBF (frozen section)	Bouin's; Zenker's
Chromaffin granules	Ferric ferricyanide reduction test	Orth's; Möller's	10% NBF; Bouin's; Heidenhain's mercuric chloride
	Gomori-Burtner methenamine silver Periodic acid-Schiff (PAS)	Orth's; Möller's	10% NBF; Bouin's; Heidenhain's mercuric chloride
Connective tissue	Reticular stains	10% NBF; Zenker's; Helly's	No picric acid fixatives
	Masson's trichrome	Zenker's; Helly's; Bouin's	NBF tissues must be post-fixed with Bouin's
	Mallory's analine blue collagen stain	Zenker's	All except preferred
Copper	Mallory's stain	Alcohol-based fixatives	Formalin
Degenerating myelin	Marchi's method	Orth's for 48 hours; 10% NBF	All except preferred
DNA/RNA	Feulgen	Ethanol	Bouin's; strong acids
Elastic fibers	Gomori's aldehyde fuchsin	10% NBF	No chromates
Fats/lipids	Nile blue sulfate	Formal calcium	All except preferred
	Osmic acid (frozen section)	10% NBF	All except preferred
	Oil red O (frozen section)	10% NBF	Zenker's; Helly's
	Sudan black B (frozen section)	10% NBF	Zenker's; Helly's
Fibrin	Mallory's phosphotungstic acid hematoxylin	Zenker's	Bouin's
	Weigert's stain for fibrin	Absolute ethanol; Carnoy's alcoholic formalin	Bouin's
Glycogen	Bauer-Feulgen	Carnoy's or Gendre's	Aqueous fixative
	PAS	Acid alcoholic formalin	Aqueous fixative
	Best's carmine	Absolute alcohol; Carnoy's	Aqueous fixative
Glycoproteins	Müller-Mowry colloidal iron	Alcoholic formalin; Carnoy's	Chromates
Hemoglobin	Lepehne's (frozen section)	Short time in 10% NBF	Zenker's
	Dunn-Thompson	10% NBF	Bouin's; Zenker's; Helly's
Hepatitis B surface antigen	Orcein		No chromates
	Aldehyde fuchsin		No chromates
Iron	Perls' stain	Alcohol-based fixatives	Acid fixatives and chromates

Continued

Table 4.2 Stains and fixatives—cont'd

Target of stain	Name or type of stain	Preferred fixative	Fixative best avoided
Juxtaglomerular cells of kidney	Bowie's stain	Helly's	All except preferred
Melanin pigments	DOPA oxidase	Fresh frozen or formalin	All except preferred
	Masson-Fontana	10% NBF	Chromates and mercuric chloride
Mitochondria		Carson-Millonig's	Dehydrants: ethanol; methanol; acetone
Mucoproteins	PAS		Glutaraldehyde
Neuroendocrine granules	Rapid argyrophil Fontana-Masson	10% NBF	Ethanol; methanol; acetone
Pancreas α, β, & δ cells	Trichrome-PAS	10% NBF or Helly's	Zenker's; Bouin's; alcohol based
	Grimelius silver	Bouin's	Glutaraldehyde
Paneth cell granules	Phloxine tartrazine	10% NBF	Acidic fixatives
Peripheral nerve elements	Bielschowski's for neurofibrils and axis cylinders	3–6 weeks in 10% NBF	All except preferred
	Bodian's for myelinated and non-myelinated nerve fibers	9 parts ethanol, 1 part formalin	All except preferred
	Nonidez's for neurofibrils and axis cylinders	100 ml 50% ethanol plus 25 g chloral hydrate	All except preferred
	Rio-Hortega for neurofibrils	10% NBF	All except preferred
	Immunohistochemistry biotin-streptavidin	Formal zinc	Alcoholic formalin
Phospholipids	Smith-Dietrich (frozen section)	Formal calcium	All except preferred
	Baker's acid hematin (frozen section)	10% NBF	All except preferred
Pituitary β cells	Congo red for β cells	10% NBF	
	Gomori's aldehyde fuchsin for β cells	Bouin's	NBF requires mordant
Spirochetes	Giemsa		Bouin's; Zenker's
	Gram's technique		Bouin's; Zenker's
	Levaditi		Bouin's; Zenker's
	Warthin-Starry	10% NBF	All except preferred
Uric acid crystals	Gomori's methenamine silver for urates	Absolute ethanol	All except preferred
	Gomori's chrome alum hematoxylin-phloxine	Bouin's	Avoid chromates

usually for approximately 48 hours; to speed fixation one or two small windows can be cut into the globe, avoiding the retina and iris, after 24 hours. After the gross description, the anterior iris and posterior optic nerve are removed with a new, sharp razor blade and the components of the globe are fixed for an additional 48 hours, or more, in buffered formaldehyde before being processed. Embedding may be in celloidin or paraffin wax. Perfusion fixation of the eye is recommended for studies of the canal of Schlemm and/or the aqueous outflow pathways.

Animal eyes may be fixed in Davidson's fixative. This fixative is rapid and gives no artifactual detachment of the retina. Fixation should not exceed 24 hours for rodents' eyes and 48 hours for larger species. Ocular globes are then transferred to 70% ethyl alcohol and processed to paraffin embedding.

Brain

The problem of fixing a whole brain is to make it firm enough to investigate the neuroanatomy and to be able to produce sections for histopathology and possible immunochemistry. Conventionally this fixation takes 2-6 weeks. Adickes et al. (1997) proposed a perfusion technique which allows all of the above to be accomplished and the report issued in 5–6 days. This method depends on the perfusion of the brain via the middle cerebral arteries, bypassing the slow rate of fixative penetration from the outside. Fixation may also be enhanced by the use of microwave technology (Anonymous, 2001; Kok & Boon, 2003; Leong, 2005). Alcoholic formalin should not be used for fixation if immunohistochemistry is to be performed using biotin-avidin (streptavidin) methods (Grizzle et al., unpublished data).

Breast

Clinical samples should be fixed in 10% NBF for a minimum of 6–8 hours, to a maximum of 72 hours and should be sliced at 5 mm intervals after appropriate gross inspection and margin designation. The time from the tissue acquisition to fixation should be as short as possible in order to prevent lysis of clinically important biomarkers such as estrogen receptors, progesterone receptors and the human epidermal growth factor receptor-2 (HER2). They should be placed in a sufficient volume of NBF to allow adequate tissue penetration. If the tumor specimen has come from a different geographical location to the laboratory, it should be bisected through the tumor on removal from the patient and sent to the laboratory immersed in a sufficient volume of NBF (Hammond et al., 2010)

Lungs

Lung biopsies are typically fixed in NBF. The lungs from lobectomy, pneumonectomy and autopsies may be inflated by, and fixed in NBF instilled under gentle pressure via the trachea or major bronchi. Such fixed lungs can be cut within 2-6 hours, and gross sections are fixed overnight, allowing sections to be processed and cut the next day.

Lymphoid tissue

Special care should be taken with all lymphoid tissue as many organisms, e.g. *Mycobacterium tuberculosis* and viruses may be present in the lymphoreticular system. There is always a possible infection risk with such cases. The lymphoid tissue is usually sliced and a representative sample of fresh tissue taken for special studies, e.g. microbiology, flow cytometry or molecular analysis. The rest of the lymph node is fixed in NBF, although some laboratories fix part of the tissue in B5 or zinc.

Testis

Biopsies of the testes are fixed routinely in NBF.

Muscle biopsies

These are received fresh and a portion is separated for enzyme histochemistry. The tissue for routine histological assessment is fixed in NBF and embedded so the fibers of the specimen are viewed in cross-section and longitudinally. After processing this is stained with H&E, a trichrome stain and Congo red if amyloid is suspected.

Renal biopsies

Renal core biopsies should be subdivided into three and each piece should contain an adequate number of glomeruli, e.g. 6-10 for light microscopy, 1-2 for electron microscopy and 3-6 for

immunofluorescence. Each portion is then preserved depending upon the method to be used for subsequent analysis:

- NBF for routine histology.
- Buffered glutaraldehyde at pH 7.3 for ultrastructural analysis.
- Snap frozen in isopentane and liquid nitrogen for immunofluorescence examination.

Useful formulas for fixatives

Gray (1954) lists over 600 formulations for fixatives and the following are the formulas for the most commonly used. Many of these formulas are based on those presented in standard textbooks of histochemistry (Sheehan & Hrapchak, 1980; Carson, 1990; Kiernan, 1999) and vary slightly from text to text.

For routine histology, 10% neutral buffered formalin (NBF) is frequently used for initial fixation and for the first station on tissue processors. NBF is composed of a 10% solution of phosphate-buffered formaldehyde. Sometimes the term 'formal' is used to refer to 10% formalin or 37% formaldehyde. Formaldehyde is commercially supplied as a 37–40% solution.

10% Neutral buffered formalin (NBF)

Tap water	900 ml
Formaldehyde (37%)	100 ml
Sodium phosphate, monobasic, monohydrate	4 g
Sodium phosphate, dibasic, anhydrous	6.5 g

Note

The pH should be 7.2–7.4. NBF purchased from commercial companies may vary widely in its aldehyde content, and commercial companies may add material such as methanol (Fox et al. 1985) or other agents to stabilize NBF preparations.

Carson's modified Millonig's phosphate buffered formalin

Formaldehyde (37–40%)	10 ml
Tap water	90 ml
Sodium phosphate, monobasic	1.86 g
Sodium hydroxide	0.42 g

Note

Deionized water can be used if tap water is hard and/or contains solids. The pH should be 7.2–7.4. This formula is reported to be better for ultrastructural preservation than NBF.

Formal (10% formalin) calcium acetate

Tap water	900 ml
Formaldehyde (37%)	100 ml
Calcium acetate	20 g

Note

This is a good fixative for preservation of lipids.

Formal (10% formalin) saline

Tap water	900 ml
Formaldehyde (37%)	100 ml
Sodium chloride	9 g

Formal (10% formalin) buffered saline

Tap water	900 ml
Formaldehyde (37%)	100 ml
Sodium chloride	9 g
Sodium phosphate, dibasic	12 g

Formal (10% formalin) zinc, unbuffered

Tap water	900 ml
Formaldehyde (37%)	100 ml
Sodium chloride	4.5 g
Zinc chloride **or**	1.6 g
(zinc sulfate)	(**or** 3.6 g)

Note

This is reported to be an excellent fixative for immunohistochemistry

Formalin buffered zinc

10% neutral buffered formalin	1000 ml
Zinc chloride	1.6 g

Mercuric fixatives

A problem with fixation in mercury solutions is that several types of pigment may combine with the mercury. These pigments are removed from sections by

using iodine treatment followed by sodium thiosulfate. They are rarely used now because of the health and safety issues related to mercury.

Zenker's solution

Distilled water	250 ml
Mercuric chloride	12.5 g
Potassium dichromate	6.3 g
Sodium sulfate	2.5 g

Note

Just before use, add 5 ml of glacial acetic acid to 95 ml of above solution. This is a good fixative for bloody (congested) specimens and trichrome stains.

Helly's solution

Distilled water	250 ml
Mercuric chloride	12.5 g
Potassium dichromate	6.3 g
Sodium sulfate	2.5 g

Note

Just before use add 5 ml of 37% formaldehyde to 95 ml of above solution. It is excellent for bone marrow extramedullary hematopoiesis and intercalated discs.

Schaudinn's solution

Distilled water	50 ml
Mercuric chloride	3.5 g
Absolute ethanol	25 ml

Ohlmacher's solution

Absolute ethanol	32 ml
Chloroform	6 ml
Glacial acetic acid	2 ml
Mercuric chloride	8 g

Note

This fixative penetrates rapidly.

Carnoy-Lebrun solution

Absolute ethanol	15 ml
Chloroform	15 ml
Glacial acetic acid	15 ml
Mercuric chloride	8 g

Note

This fixative penetrates rapidly.

B5 fixative

Mercuric chloride	12 g
Sodium acetate	2.5 g
Distilled water	200 ml

Note

Add 2 ml of formaldehyde (37%) to 20 ml of above solution just before use. It is frequently used for bone marrow, lymph nodes, spleen, and other hematopoietic tissues.

Dichromate fixatives

There is a variation among the names attributed to the formulas of dichromate fixatives but not in the formulas themselves. Time of fixation (24 hours) is critical for dichromate fixatives. Tissue should be washed after fixation and transferred to 70% ethanol. Failure to wash the tissue after fixation may cause pigments to be precipitated. Extensive shrinkage may occur when tissues are processed to paraffin blocks.

Miller's or Möller's solution

Potassium dichromate	2.5 g
Sodium sulfate	1 g
Distilled water	100 ml

Möller's or Regaud's fluid

Potassium dichromate	3 g
Distilled water	80 ml

Note

At time of use add 20 ml of formaldehyde (37%).

Orth's solution

Potassium dichromate	2.5 g
Sodium sulfate	1 g
Distilled water	100 ml

Note

At time of use add 10 ml of formaldehyde (37%).

Picric acid fixatives

Many picric acid fixatives require a saturated aqueous solution of picric acid. Aqueous picric acid 2.1% will produce a saturated solution, and 5% picric acid a saturated solution in absolute ethanol. Bouin's

solution is an excellent general fixative for connective tissue stains. The yellow color can be removed with 70% ethanol, lithium carbonate or another acid dye separately, or during the staining sequence. The solution destroys membranes, therefore intact nuclei cannot be recovered from fixed tissue and there may be extensive shrinkage of larger specimens.

Bouin's solution

Saturated aqueous solution of picric acid	1500 ml
Formaldehyde (37%)	500 ml
Glacial acetic acid	100 ml

Note

Fixation time should not be more than 5 days.

Hollande's solution

Distilled water	1000 ml
Formaldehyde (37%)	100 ml
Acetic acid	15 ml
Picric acid	40 g
Copper acetate	25 g

Note

A useful fixative for gastrointestinal biopsies and endocrine tissue. Specimens are washed before exposure to NBF.

Dehydrant fixatives

Dehydrant fixatives act to remove free and bound water, causing a change to the tertiary structure of proteins so that they precipitate, leaving the nucleic acids relatively unchanged. Ultrastructure is also destroyed due to the extraction of lipids and excessive shrinking of tissue components may occur after more than 3–4 hours of fixation. Each of the following dehydrant fixatives can be modified by adding other chemicals to produce specific effects.

- Absolute ethanol
- 95% ethanol
- 70-95% ethanol
- Methanol
- Acetone.

Methanol is useful for touch preparations and smears, especially blood smears. Many alcohol mixtures undergo slow reactions among ingredients upon long term storage so most alcohol-based fixatives should be prepared 1–2 days before use. Acetone fixation should be short (1 hour) at 4°C on small specimens. Acetone produces extensive shrinkage and hardening, and results in microscopic distortion. It is used for immunohistochemistry, enzyme studies and in the detection of rabies. Cold acetone is especially useful to 'open' the membranes of intact cells, i.e. those grown on coverslips or microscope slides to facilitate entrance of large molecules, e.g. antibodies for immunohistochemical studies. 'Trade secret' ingredients stabilize commercial formulations.

Clarke's solution

Absolute ethanol	60 ml
Glacial acetic acid	20 ml

Note

This solution produces good general histological results for H&E stains. It has the advantage of preserving nucleic acids whilst lipids are extracted. A short fixation is recommended and tissues are transferred to 95% ethanol following fixation.

Carnoy's fixative

Acetic acid	10 ml
Absolute ethanol	60 ml
Chloroform	30 ml

Note

Carnoy's fixative is useful for RNA stains, e.g. methyl green pyronine and for glycogen preservation. It shrinks and hardens tissues, hemolyzing red blood cells. It may destroy the staining of acid-fast bacilli. It is useful in cytology to clear heavily blood-stained specimens.

Methacarn's fixative

Acetic acid	10 ml
100% methanol	60 ml
Chloroform	30 ml

Note

Causes less hardening and less shrinkage than Carnoy's, but with the same pattern of staining.

Dehydrant cross-linking fixatives

Compound fixatives with both dehydrant and cross-linking actions include alcohol-formalin mixtures.

Alcohol-formalin fixation or post-fixation can be advantageous in large specimens with extensive fat. Lymph nodes can be detected more easily in specimens with alcohol-formalin fixation due to the extraction of lipids and to texture differences compared with tissues fixed in NBF. The preparation of alcohol-formaldehyde solutions is complex, especially buffered forms. It is probably best to purchase commercial preparations of buffered alcohol-formaldehyde. For use in post-fixation (e.g. after 10% NBF), Carson (1990) recommends the following formula:

Absolute ethanol	650 ml
Distilled water	250 ml
Formaldehyde (37%)	100 ml

Note

Carson recommended this formula because it was noted that the concentration of ethanol should be less than 70% to prevent the precipitation of phosphates in 10% NBF saturated tissues. For initial fixation the following formulas can be used:

Alcoholic formalin	
Ethanol (95%)	895 ml
Formaldehyde (37%)	105 ml

Alcohol-formalin-acetic acid fixative	
Ethanol (95%)	85 ml
Formaldehyde (37%)	10 ml
Glacial acetic acid	5 ml

Note

Methanol may be substituted for ethanol with care. Similarly, various mixtures of ethanol, acetic acid and formalin may be used.

Alcoholic Bouin's (Gendre's solution)

This fixative is similar to Bouin's except it is less aqueous and there is better carbohydrate retention, particularly glycogen. Fixation should be between 4 hours and overnight, followed by washing in 70% ethanol and then several washes in 95% ethanol. This is the one alcoholic fixative which improves with aging (Lillie & Fullmer, 1976).

Gendre's solution	
95% ethanol saturated with picric acid (5 g per 100 ml)	800 ml
Formaldehyde (37%)	150 ml
Glacial acetic acid	50 ml

Note

To increase the effectiveness of alcoholic Bouin's, if there is no time for aging, the following formula has been recommended (Gregory, 1980):

Equivalent to aged alcoholic Bouin's	
Picric acid	0.5 g
Formaldehyde (37%)	15 ml
95% ethanol	25 ml
Glacial acetic acid	5 ml
Ethyl acetate	25 ml
Tap water	30 ml

Alternative alcoholic form of Bouin's solution	
Bouin's solution (page 58)	75 ml
95% ethanol	25 ml

Note

This solution is excellent for lymph nodes (24 hours) and for fatty tissue (48 hours).

Rossman's solution	
Tap water	10 ml
Formaldehyde (37%)	10 ml
Absolute ethanol	80 ml
Lead nitrate	8 g

Note

Fix for 24 hours at room temperature. This is a good fixative for connective tissue mucins and umbilical cord.

Other fixatives

Fixative for metabolic bone disease	
Phosphate buffer (page 528)	900 ml
Formaldehyde (37%)	100 ml
Adjust pH to 7.35.	

Davidson's fixative for eyes	
95% ethyl alcohol	300 ml
Formaldehyde (37%)	200 ml
Glacial acetic acid	100 ml
Distilled water	300 ml

Note

This fixative gives good nuclear details and may also be used to fix hematopoietic organs as well as rodent testes (fix for 24 hours).

Fixation for fatty tissue	
Bouin's solution (page 58)	75 ml
95% ethanol	25 ml

Note

May require up to 48 hours for good sections of lipomas or well-differentiated liposarcomas.

Zinc-Tris fixative	
0.1M Tris buffer, pH 7.4	1000 ml
Calcium acetate	0.5 g
Zinc acetate	5.0 g
Zinc chloride	5.0 g

Notes

Mix to dissolve. The final pH will be approximately 6.8. Do not readjust the pH, as this will cause the zinc to come out of solution. Fix for 24–48 hours. This fixative is useful for the preservation of fixation-sensitive antigens in paraffin-embedded sections (Beckstead, 1994). It is a recommended fixative for the detection of CD4 and CD8 on murine tissue.

Acknowledgment

We would like to thank all the previous contributors to this chapter for their scientific input and Dr Catherine Cannet for her review of the chapter for this edition and her updates.

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5 The gross room/surgical cut-up including sample handling

S. Kim Suvarna

Introduction

The initial dissection and preparation of any specimen for histological/microscopic analysis involves more than simply the transcribed macroscopic description and sampling of the specimen. Whilst the dissection and laboratory area are often perceived as the two key elements of the department, it must be clearly understood that there are many steps which follow specimen receipt, interfacing with the dissection room, that directly affect case handling. Some are specific to tissue selection and handling and others are clearly support roles.

It should be self-evident that a good laboratory will be adequately staffed by appropriately trained scientific/medical and support staff (secretarial, medical laboratory assistants, administration, etc.), and these staff interface at multiple levels with pathological sample handling. It has been demonstrated on many occasions that a poorly staffed department, whether in personnel and/or training, will perform suboptimally, to the detriment of sample analysis and therefore patient care.

Safety first and last

The histopathology department is rich in hazards, e.g. infection/biological, chemical and radiation. There are also various risks reflecting the range of materials used to store, process and analyze tissues. These may be toxic, flammable, allergenic, carcinogenic and electrical. The presence of sharp cutting implements, complex machinery and the movement of the specimens around the laboratory heightens all of these risks. Staff need to be fully trained to be aware of all of these potential hazards and capable of operating safely in this environment. Every laboratory should have accessible and clear standard operating procedures (SOPs, see Chapter 1), many of which will reflect national/international guidelines (websites 1–3). Ongoing safety education as part of continuing professional development is required, and caution should be employed at every step of specimen handling for safe laboratory practice (websites 2 and 4).

Specimen reception

A separate room is required for specimen reception which acts as the interface between non-laboratory hospital staff, other visitors and the pathological laboratory. The area must be equipped with appropriate easily cleaned benching, adequate lighting, good ventilation, safety equipment, disinfectants, absorption granules and protective clothing. In the event of specimen spillage, e.g. body fluids, fixative leakage or other mishap, the immediate response by staff in this area will limit any potential local health risk and prevent risk to other laboratory personnel.

The key point of this room is to receive samples safely and securely. Any new specimen should have its identity confirmed and assigned a unique laboratory specimen identifier, usually a complex number. Mapping of the specimen identifier against the clinical request form is mandatory, along with checking of appropriate clinical details mentioned against the specimen. Corroborative data, in the form of the hospital number/registration index, national patient identifier number, the full name, date of birth and address are valid ways of verifying the identity of any specimen. Multiple sources of cross reference are always advocated, and if there is any doubt with

regard to the probity of a specimen then it should not be passed onwards until the clinician concerned has confirmed all the appropriate details.

In many situations the two-person rule is best followed with two independent laboratory practitioners verifying the various details of the specimen at all the different stages of examination. Confirmation of a minimum of three unique identifiers (as detailed above) is advisable. Once validated and identified, the case, usually labeled with a self-adhesive bar or quick response (QR) code label, can be passed to the dissection room for examination, specimen description and block sampling.

The usual numerical method of specimen identification is simply the year, expressed in two digits, with a sequential numbering system starting with one (1) and proceeding up to the final specimen of each year. There may be a check digit, often in the form of a letter applied, but this simple system allows surgical pathology samples to be processed with ease and to be correlated against paraffin blocks, photographs and other tests (see below). Thus, case 2345L/17 is the two thousand, three hundred and forty-fifth sample of the year 2017. The letter L is a computer check datum to verify that the numerical data is valid.

Particular attention must be paid to cases with unusual or common names. Names which have a variety of different spellings and any specimens which have incomplete information should be carefully considered before being accepted. Those with imprecise verification data and poorly handwritten data should not be accepted.

In some cases, multiple specimens from a single patient may be received on the same day for analysis. Some laboratories prefer to annotate each sample with a separate number. However, a single laboratory number may suffice, but with sub-parts of the specimen being separately designated, e.g. sample A, sample B, etc. Within this framework, if multiple blocks are taken from a sub-part of the specimen then these can be designated with individual numbers or letters in a similar ascending fashion. Thus, a gastrectomy sample with two separate lymph node groups and the spleen could have one case number, 2345L/17, multiple sub-part specimens (A, B, C) and multiple blocks (1, 2, 3, etc.) which can be correlated against the surgeon's operative dissection. Using the number described above, e.g. the spleen in this case could be designated 2345L/17. C.2 (C. indicating the sub-part of the third sample = spleen and the block number = 2). At this stage the sample may now confidently be passed into the dissection room.

Barcodes and QR codes can be used to assist material handling within the laboratory, but in general terms many laboratories still have paper request forms which will accompany the specimen as it passes throughout the laboratory and towards final report production (see Chapter 11, Automation in the histology department). The departmental computer system can be set to track specimen movement through the laboratory from its receipt to the final histopathologist report authorization.

Surgical cut-up/specimen dissection/grossing

The ideal layout of this room is a matter of debate, varying between different laboratories and pathologists' needs. Numerous design solutions exist around the general principles of a histology laboratory (Rosai, 2011; Lester, 2010; Cook, 2006, websites 2, 4), but it is imperative that the dissection area must have good electrical or natural lighting, good ventilation and non-absorbent wipe-clean surfaces. Within the area there must also be appropriate protective clothing for the laboratory personnel including gloves and other equipment, e.g. photography, tissue macerators and disposal bins.

The dissection room should be a comfortable environment permitting undisturbed work by the pathologist and support technical staff (Fig. 5.1). Given that the range of specimens received in most laboratories is wide, the technical staff will have to be familiar with the various requirements of different specimens which guide their subsequent handling and pathological preparation.

It is a matter of preference whether the operators within this environment sit or stand, and ideally both options should be available to suit individual staff members. Modern dissection areas often have integrated dissection desks, enclosed fluid/fixative

Fig. 5.1 A pathology dissection station with a downdraft ventilated bench and clear dissection zone. Note the well-lit and ergonomic layout for the grossing pathologist and the technician support. (Grateful thanks are expressed to Dr Caroline Verbeke and Mr Jonathan Sheriff for their assistance and consent for the illustration.)

feeds and laminar down-draft ventilation (website 5) protecting all the staff from formalin vapor and hazardous fluids. All tools and materials should be ergonomically accessible.

Thinking before dissection

Prior to fixation it may be relevant to reserve some fresh tissue from the specimens for microbiology assessment by placing into appropriate culture media and/or electron microscopy which requires glutaraldehyde fixation. Fresh tissue may be taken for frozen section immediate analysis. Unfixed tissue can also be taken for DNA extraction, cytogenetics and molecular pathology techniques. The latter is becoming increasingly common and important in the arena of personalized therapy by reflecting the tumor genotype and characteristics. Other specialized tests, e.g. enzyme assay and mass spectroscopy may also require tissue retention before standard formalin fixation.

Some specimens are only examined macroscopically, possibly with photography and other physical techniques. Examples include various mechanical/prosthetic implants, foreign bodies, bullets, gallstones and medical devices. These must be dealt with according to the needs of the specific request/case. It should be noted that some specimens may require retention for a prolonged period of time, e.g. in forensic/criminal investigation cases.

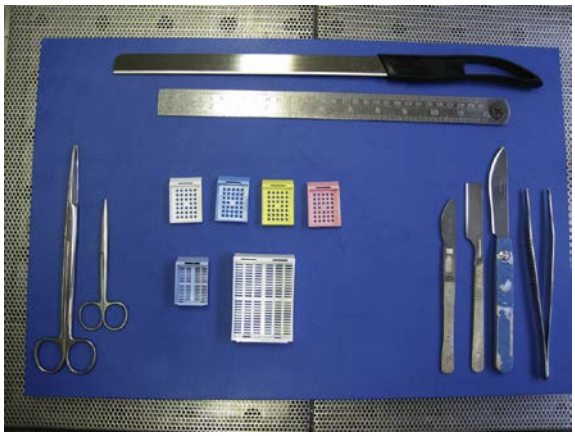

Fig. 5.2 Cut-up/grossing tools. A range of small and large bladed tools are advocated along with forceps, ruler and a fluid-resistant dissecting surface. An appropriate measure and access to photography are needed. Varying sizes of cassette (center) are available, in a range of colors and sizes, to permit handling of varying amounts of sample and also to indicate handling issues which follow tissue processing.

At this point, if no preliminary sampling is performed, the specimen is usually fixed, by immersion into formalin. This process fixes (see Chapter 4) the sample, allowing storage for a prolonged period without tissue degradation. Some samples need fixation and then decalcification in ethylenediaminetetraacetic acid (EDTA) (see Bone, Chapter 17).

Case handling

This dissection/blocking/grossing/cut-up facility must have an appropriate storage area immediately to hand allowing clearance of already-examined samples promptly, preventing the dissecting area becoming cluttered.

The individual choice of dissecting tools will reflect the type of specimen being considered (Fig. 5.2). However, a range of sharp cutting blades is advised, enabling the dissector to deal with small

Fig. 5.3 Tissue blocks are placed into cassettes. Note the samples should not fill the cassette, and must permit room for processing fluid circulation. The orientation of the blocks is enhanced by a sponge securing the specimens in sequential position and a colored agar marker allows designation of the order of slices taken. The samples have been marked with different colored inks to permit designation of the sidedness of the samples and the resection margins.

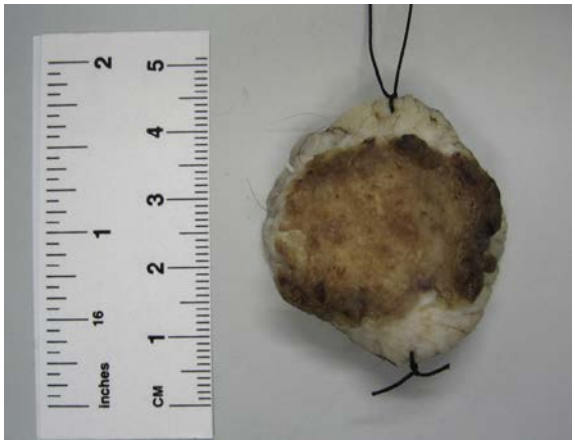

Fig. 5.4 A medium-sized skin sample is seen with a central lesion. This could be described as 'A skin ellipse x by y by z mm depth is seen with an orientation suture, designated 12 o'clock. The sample shows a central yellow-brown nodule z mm which is k mm clear of the closest margin'. It is sectioned into parallel slices and then placed into a cassette (Fig. 5.3).

specimens through to complex and large resections. Long knives are particularly useful for obtaining full transverse sections of whole organs, e.g. lungs and liver. The smaller blades are useful for precise trimming of tissues. The blade must be sharp if one is going to confidently sample the tissue appropriately to produce blocks of the correct thickness and shape. However, before any knife is put to the specimen, it is emphasized that the tissue specimen must be well fixed.

Forceps and absorbent cloths should be available adjacent to the work area. The blocks of tissue taken should not completely fill the cassette as this impedes the later processing fluid accessing all of the tissue (Fig. 5.3). Tissues are normally put into standard tissue cassettes which are usually made of plastic and now conform to a variety of size standards across the developed world. Most standard blocks allow a sample of about 20 × 20 × 3 mm thick tissue to be contained and processed adequately. There is variation in cassette size which does allow larger blocks to be selected (Fig. 5.2). This is particularly useful for histological examination of large surgical resections where the global geography of the specimen is needed for analysis, e.g. complex rectal cancer resections, radical prostatectomy and autopsy lung tissue for industrial disease.

However, some general rules can be developed to specimen handling and sampling. The specimens should be analyzed with only one pot open at any one time. The request and specimen identity should be checked, ideally by two persons, the dissector and their assistant. The sample should be described in terms of the nature, shape, size and also any defining characteristics. This means that small biopsies, e.g. endoscopic mucosal samples, may simply be afforded a simple descriptor in the form of the number of pieces and the size (SI units, usually mm) of the largest piece of tissue. An example could be 'three pieces of brown tissue, the largest 3 mm diameter'.

Medium and large specimens (Fig. 5.4) need more detail and a careful description of the various anatomical components, together with identification of macroscopic landmarks, orientation markers/sutures and any lesion(s) if relevant. The background tissues, beyond the lesion under consideration, also require description.

The sampling of any large case/resection (Fig. 5.5) should follow local and national guidelines in order to provide the relevant information for

Fig. 5.5 A lung lobectomy sample, sliced to show the hilar and mediastinal plane of resection, highlighting tumor adjacent to the margin. Block sampling at this interface and background tissue sampling against standard protocols will allow full analysis. Note the numbered cassette along with the ruler for full case identification and analysis.

the subsequent clinical management of the patient (website 6).

The macroscopic description is usually dictated for subsequent secretarial transcription, or occasionally can be simply written down for typing later. Canned or proforma reports may be of value to standardize the approach to samples, but those in the dissection suite must be capable of some adaptation since no two cases are identical.

Once the case sampling has been finished, any excess material should be kept generally for 1-3 months as further sampling may be needed. Storage should be close to the dissection area on sturdy shelving with good ventilation and easy access (Fig. 5.6). The shelves should not be at high level

If one knows beforehand of additional tests which will be automatically required on a specimen, e.g. a renal transplant biopsy (requires multiple levels, ancillary histochemical stains and immunohistochemistry tests), then different color cassettes or markers can be used in order to designate standardized additional actions which should follow as an automatic laboratory consequence (Fig. 5.2). The different colored cassettes may also indicate the types of sample contained, as well as the urgency of any specimen.

Fig. 5.6 A storage area for cases following dissection and sampling. There is sturdy shelving with non-crowded samples. The safe stepping-stool device may assist review of shelf contents, but it is vital that sample storage shelving is not at high level, as this could pose a risk in taking samples on/off shelves with potential spillages onto the laboratory staff.

Following dissection, the residual tissues must be stored in a ventilated secure archive format, and waste materials must be disposed of according to local/national health and safety regulations (websites 4, 5, 8).

Photography

Photographing the macroscopic specimen, whole or during the dissection, is particularly important in cases of complex surgical excision, e.g. Wertheim's hysterectomy, pneumonectomy and localization samples. It may also be of use in later analysis/case discussion (Fig. 5.5). Digital photography with hand-held basic devices has been a major bonus to the laboratory superceding film-based photography. The opportunity to enhance the patient record

has greatly improved all pathology disciplines and allowed retrospective case reviews. Specialist and professional photography may still be required however for cases which may be used in visual teaching presentation, journal/book publication or in a medico-legal situation. Consideration of the potential to recognize a patient's sample should be made, with some guidance existing on the subject (website 7).

Specimen dissection plans

Small samples

Small biopsy samples rarely need dissection and can be processed as they present i.e. whole, embedded and then sectioned. In some cases, orientation of the specimen in the block can be facilitated by means of a dissecting microscope or magnifying lens, e.g. when considering morphological abnormalities of small bowel biopsies. However, the majority of small biopsies can be adequately examined at multiple levels allowing the pathologist to mentally reconstruct the three-dimensional quality of the tissue during microscopic examination.

These small samples may benefit from being placed in a nylon bag, between metal disks with fine mesh, or wrapped in paper in order to prevent the samples falling through the cassette perforations and being lost. Eosin can be used as a marker for small samples in order to highlight them on the background of paper, embedding bench or equivalent. It is recommended that a count of the small tissue biopsy fragments is always taken at the description/grossing stage in order to verify that all the tissue has survived processing prior to section cutting.

Core biopsies

These are treated in a similar manner to small biopsies, although their embedding requires being laid out in longitudinal fashion so that the plane of section cuts along the majority of the tissue. At this stage, larger cores with diameters of 4–5 mm or greater may benefit if divided into two halves along the long axis, increasing the tissue available to review histologically. However, this risks tissue damage and can be time-consuming and difficult. The alternative solution may be to simply provide multiple levels with retention of tissue in between the levels for further analysis. Multiple cores, even if in the same specimen pot, often require each core to be placed in an individual cassette in order to prevent tissue loss during 'trimming-in' after processing.

Skin biopsies

These include simple punch biopsies (handled akin to cores) and shave biopsies which should be mounted on edge in order to provide an adequate view of the epidermis, dermis and subcuticular layers. A marker item placed into the cassette, e.g. plastic bead or colored paper will alert such samples to embedding personnel. Alternatively, some laboratories use cheese paste to help maintain specimen orientation (Tripathi et al., 2008). The protein in the paste helps hold the tissue orientation during processing (Fig. 5.7).

Skin samples may include the more complex intermediate and large specimens. These are required for removal of large defined lesions and radical skin cancer resections which may include deep soft and/or bony tissues.

The intermediate and larger samples of skin are often presented as an ovoid or ellipse piece of skin and subcutis, usually with a central lesion (Fig. 5.4). These must be described in terms of the width and breadth of the specimen together with a depth. After the fixed tissue is sectioned, usually at 3 mm intervals along the specimen the lesion characteristics, e.g. nodule, ulcer or papule, color and the distance to the margins should be recorded. Photography may be of value. Specimens of these skin resections are often best managed in serial transverse section, with Indian ink or another dye being applied to different surfaces in order to confirm the orientation or boundaries of the specimen (Fig. 5.3). Markers can be placed into cassettes in order to confirm pieces of tissue with orientation markers, although it is generally found that specimens start with small apical transverse sections through to the broadest point across the waist of the specimen and then taper off towards the other end.

Fig. 5.7 Cheese paste is seen holding the thin fragment of inked skin on edge and in position securely. The cheese protein matrix will survive tissue processing!

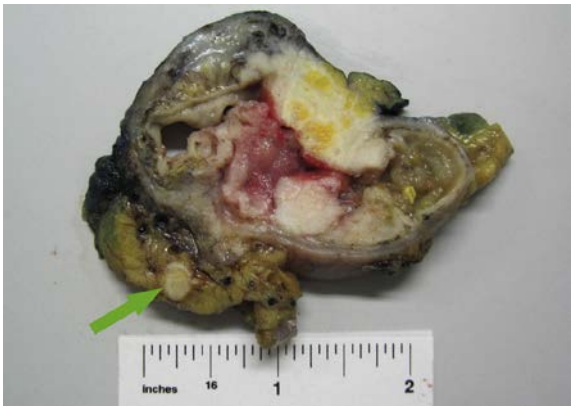

Fig. 5.8 A large bowel specimen is seen with anatomical complexity requiring a good description and multiple blocks to be taken for full analysis. This sample shows the resection, opened to visualize the cancer. The specimen has been photographed to facilitate understanding of the local resection margins and the serosal surface (inked). The entire tumor and local bowel can be blocked into a large cassette, if desired. A lymph node (arrowed) is clearly involved by tumor in the fatty serosal tissues, but the fatty mesentery can be removed for fat-clearance nodal identification and analysis.

Large resections of skin with soft tissues may require photography and then targeted block sampling, as it is unreasonable to embed and section all of it. This should also allow the appropriate assessment of any tumor or lesion with deep and lateral margins. Multiple blocks of the pathology within the specimen are normally taken in order that any disease variations within the lesion will be identified. These samples, and indeed their smaller counterparts, should be blocked to permit reporting against national and/or international standards.

Bowel specimens

Apart from endoscopic gastro-intestinal biopsies, bowel samples are generally medium and large tissue resections which can originate from anywhere along the length of the gastrointestinal tract, e.g. esophagectomy, gastrectomy and partial colectomy. They are best sampled with multiple (3 or more) blocks of any lesion in relation to the adjacent mucosa, wall and serosal aspect of the tissues (Fig. 5.8), although large geographic ('jumbo') blocks can be employed. The margins often need inking and the background tissues including resection margins are often included as part of the relevant dissection protocol (Allen, 2013; Allen & Cameron, 2012, website 6). Particular attention is paid to the lymph nodes which can either be manually dissected in groups, or be identified from fat-clearance protocols (Prabhudesai et al., 2005) as described below. It is vital that the nodes are assessed in terms of their proximity to the lesion along with their different 'level' stages. Many cases require consideration of the high tie, i.e. the most proximal lymph node in the resection (or equivalent).

Fat clearance

Finding lymph nodes within a large amount of fatty mesenteric or soft tissue can be particularly problematic when trying to isolate individual small nodes. The ability to remove the adipose substrate from any specimen will lead to an enhanced rate of

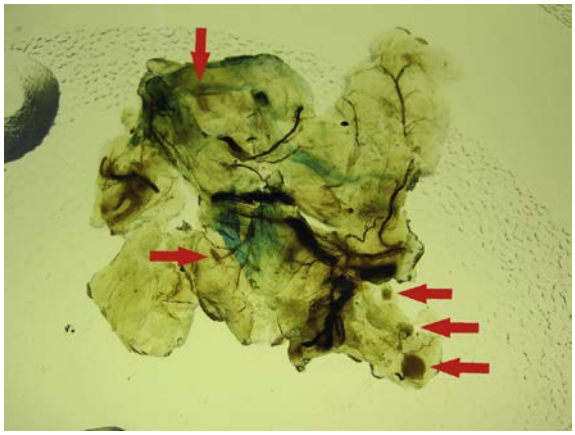

Fig. 5.9 Following fat clearance, the transilluminated sample is searched for nodes (arrowed). The nodes are then extracted and placed into smaller cassettes for routine histology assessment after the final stage of processing and embedding.

node detection and thereby sampling. This will raise the accuracy of tumor staging and patient management. One aspect of the histological tissue handling in the cut-up room allows such node identification (Prabhudesai et al., 2005). In this process, the fatty tissue is usually sliced into 5 mm fragments and placed into large cassettes which increases the access of solvents to the specimen. Fat removal occurs as part of the processing of tissues, but the blocks of fatty parenchyma are normally removed from the processing chamber before the tissues are impregnated finally with wax. At this stage the lymph nodes can be readily palpated and identified by transillumination of the tissue sample from below (Fig. 5.9). The sampled nodes can then be placed back in the tissue processor in a smaller cassette, with normal embedding, sectioning and staining to follow.

Lung tissues

Aside from small bronchi, transbronchial and pleural biopsies, the lung samples commonly received for histology are localized, wedge biopsies or lobectomy and pneumonectomy specimens. There are also occasional radical resections which can include, e.g. pleuro-pneumonectomy, lobectomy and the chest wall. The background pleura and lung must be evaluated along with any lesions as required in standard proforma sampling protocols (Allen, 2013; Allen & Cameron, 2012, website 6). In general terms, multiple blocks for any tumor (4 or more) along with sampling of the pleural/mediastinal/bronchial margins are needed. Nodes are often presented in groups separately, although careful dissection of the hilar tissues should allow further node harvest from these tissues (Fig. 5.5).

Gynecological samples

Common samples include fragments of endometrium removed by curette or equivalent and small punch biopsies. These are generally embedded whole and processed in one cassette. More complex samples, e.g. cone biopsies from the cervix, need appropriate inking of margins and orientation, often in a serial block fashion across the specimen with photography. This allows the three-dimensional assessment of dysplasia or invasive neoplasia in relation to the various surgical margins. Uterine samples are usually sampled in terms of the cervix if included, endometrium and myometrial tissues together with some representative sampling of common benign lesions, e.g. fibroids (leiomyomas). Sampling will be guided by local practice together with national guidelines (Allen, 2013; Allen & Cameron, 2012, website 6). Dysplastic and malignant lesions often require multiple blocks including resection margins and careful examination of related lymph nodes which are usually presented, and therefore blocked, separately. Specific tissues such as tubes and ovaries should follow similar standard guidelines in terms of the sampling pattern, number of blocks and related tissue samples. It is emphasized that pluripotential differentiation of tumors within the female genital tract requires multiple blocks of a tumor to be taken for full analysis.

Breast resections

These are also common surgical resections, usually with the need for inked margins in relation to the orientation of the specimen. Multiple blocks of the tumor are usually required. The background tissues should also be assessed at multiple points and the lymph nodes, if present, are often examined in a tiered or grouped fashion. This allows the tumor spread to be assessed by identifying the size of the nodes involved and the furthest node from the primary which has

been affected. Fat clearance may be required to capture all the nodes in the axillary tail.

Soft tissue resections

These vary but should be examined with multiple blocks of tissue, background parenchyma and the margins. Some experts advocate 1 block for every 10 mm diameter of tumor up to 10 blocks, although more may be required on occasions. Careful slicing and examination of the specimens macroscopically will allow sampling and consideration of all the peripheral boundaries. Furthermore, given the pervasive nature of soft tissue tumors this widespread sampling is usually required. Tumor sampling before fixation for molecular and/or genetic analysis may be required.

Other samples

This chapter is not designed to be able to discuss all possible resections and specimen subtypes and the groups above are illustrative only. Several of the references below will assist the reader with a fuller range of dissection approaches, alongside publications from governing bodies and national organizations which have produced protocols for the analysis of specimens (Allen, 2013; Allen & Cameron, 2012, website 6).

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6 Tissue processing

Dee Wolfe

Introduction

Proper handling of tissue specimens is critical to ensure that an accurate diagnosis is obtained from patient tissue samples. Whilst technological advances have streamlined processing, the principle steps remain the same: fixation, dehydration, clearing and infiltration. Regardless of the methodology, tissue samples requiring processing need to be placed in fixative as soon as possible after excision from the patient. This first step is essential to prevent autolysis, which could destroy diagnostic elements, and to prepare the tissue for the rigors of the reagents used in subsequent processing steps. This chapter will serve as a patient to pathologist guide for preparing tissue samples which ensures an accurate diagnosis with the best treatment outcomes for the patient.

Specimen tracking

To ensure accurate specimen tracking and maintain patient confidentiality, all tissue received in the laboratory must be given a unique identifier. Typically, an alphanumeric code is assigned to each sample. This code follows the specimen on its journey from the moment it is received in the laboratory to the final pathology report. Whilst chemical-resistant pens, pencils, slides and labels have been the standard, new technology utilizing barcodes, quick response (QR) codes and character recognition are readily available in most laboratories. Automated pre-labeling systems which permanently etch or emboss tissue cassettes help reduce human error. Many of these systems can track the particular run a block was processed on, offering greater information for auditing purposes. This type of data acquisition also assists in maintaining LEAN processes. Many histology laboratories in the USA are incorporating LEAN principles adapted from the Japanese manufacturing industry to streamline workflow, eliminate waste (either in materials or time), and foster a system of continuous improvement which will deliver a better "product", i.e. quality tissue preparations which will allow faster diagnosis for improved patient outcomes. Regardless of whether an automated or manual labeling system is used, adequate policies and procedures must be in place to ensure positive identification of the tissue blocks and slides during processing, diagnosis and filing.

Factors influencing processing

When tissue is immersed in fluid, an interchange occurs between the tissue's internal fluid and the surrounding solution. The rate of fluid exchange, or diffusion, is dependent upon the tissue size and density as well as the physical properties and concentration of the processing reagents. Several factors influence the rate at which diffusion occurs.

Viscosity

A solution with low viscosity has smaller sized molecules and a faster penetration rate. Conversely, solutions with larger molecules have high viscosity and the exchange rate is slower. Most of the solutions used in processing, dehydration and clearing have similar viscosities; embedding media however have varying viscosities. Melted paraffin wax, for example, has a low viscosity which enhances the impregnation rate.

Agitation

This increases the flow of solutions around the tissue. The mechanism for agitation in automated processors incorporates either vertical or rotary oscillation or pressurized removal and replacement of fluids at timed intervals.

Heat

An increase in temperature improves the fluid exchange and penetration rate, but this must be used sparingly to reduce the possibility of producing morphological heat artifact. Excessive exposure to heat can cause shrinkage and hardening of the tissue which negatively affects subsequent staining and immunohistochemistry.

Vacuum & pressure

These both increase fluid mobility, thus increasing the infiltration rate and decreasing the time necessary to complete each processing step. Vacuum aids the removal of air pockets in porous tissue, e.g. lung.

Processing solvent contamination

The number of blocks on each run, tissue type, size, frequency of the runs, use of sponges and cross-contamination of processor solvents will influence how often solutions should be rotated between stations or changed to maintain processing quality.

Tissue processing stages

- Fixation – prevents autolysis and stabilizes tissue to maintain cellular structure.
- Dehydration – removes water and unbound fixative from the tissue.
- Clearing – displaces dehydrating solutions, making the tissue components receptive to the infiltrating medium.
- Infiltration – permeates tissue with a support medium.
- Embedding – orientation of the tissue sample in a support medium to create a tissue "block" suitable for sectioning.

Fixation

The first and most critical step in specimen handling is preservation or fixation of the tissue sample. Fixation denatures proteins rendering the cell and its components resistant to further autolysis. Complete fixation also allows the tissue to withstand the negative effects of subsequent processing reagents. Although samples are typically received in fixative, it is best to begin processing with a fixative station as the natural diffusion of water from the tissue will have diluted the fixative in the specimen container reducing the fixative's efficacy.

Inadequately fixed tissue may exhibit zonal fixation due to the dehydrating solutions completing the fixation process towards the interior of the tissue. This may result in altered morphology and affect the staining characteristics of the tissue. The size and type of specimen in the tissue cassette determines the time needed for complete fixation and processing to occur. The tissue should be dissected to 2–4 mm in thickness and trimmed to a size that allows complete flow of reagents around the tissue in the cassette. Ideally tissue should be separated according to size and/or type, and processed using different schedules. The most commonly used reagent for the fixation of histological specimens is 10% neutral buffered formalin (NBF). Alcoholic formalin can be used to accelerate the penetration rate of the fixative and to shorten the time needed for complete fixation. Zinc formalin solutions are used for their ability to produce sharp nuclear detail and enhance staining capability. Non-formalin fixatives with glyoxal as the active ingredient have gained popularity for their added safety benefit (see Chapter 4).

Post-fixation treatment

Specimens fixed in alcoholic fixatives should be followed with alcohol to prevent re-introduction of water to the tissue specimen. The alcohol concentration will be dependent on the alcohol concentration of the fixative.

Picric acid fixatives, e.g. Bouin's, will color the tissue bright yellow. A rinse of the tissue in 50-70%

alcohol for 4-6 hours (Luna, 1968) will remove excess fixative. Complete removal of the yellow color is essential to prevent the color from interfering with staining. This can also be accomplished by treating the cut tissue section on the glass slide with a dilute carbonate solution (see Stainsfile website, 2016.)

A few drops of 1% eosin can be added to the specimen container 30 minutes prior to processing to assist in visualizing small tissue fragments during embedding. The pink color of the tissue remains during processing, but washes out during subsequent staining. Incomplete removal of the eosin can interfere with fluorescent procedures.

Dehydration

Dehydration displaces the residual fixative as well as cellular water. Water is found in the tissue in two forms, free and bound. The bound water molecule is an integral part of the macromolecules of the cell. Correct dehydration schedules during tissue processing should only remove the free water, leaving the bound water intact. Graded alcohols are used in dehydration to remove free water and keep the bound water in place. When tissues are exposed to heat or excessive time in the higher grade alcohols (95% or 100%), bound water is removed. The removal of the bound water will produce over-processing artifacts such as shrinkage, 'parched earth' effect and abnormal staining, as well as dry, brittle tissues during microtomy. For this reason, specimens are best processed through graded alcohols of increasing concentration. Incomplete dehydration will impair the penetration of the clearing reagents into the tissue, leaving the specimen soft and non-receptive to paraffin wax infiltration. Ethanol, reagent alcohol and isopropanol all have a strong history of working well in tissue processing. Glycol ether dehydrants are an effective alternative to alcohols in tissue processing. Unlike alcohol, glycol ethers are unable to act as a secondary fixative but their chemical properties prevent removal of bound water so over-processing is prevented. Due to their gentle nature they are used undiluted (Feldman et al., 2014).

Clearing

Clearing agents must be miscible with both the preceding anhydrous alcohols/dehydrants to effectively remove them, and with the ensuing paraffin wax to allow complete infiltration. A good clearant will also dissolve lipids which can impede the wax penetration. Many of the historical solvents are not suitable for histology processing today due to their extremely hazardous nature and/or harshness to the tissue, e.g. carbon tetrachloride and chloroform. Chloroform does not harden tissue but was not approved for use on earlier closed tissue processors because it could dissolve the plastic components. Whilst it is on the approved reagent list for newer processor models, the downfall is that chloroform is carcinogenic.

Today, xylene is the most widely used clearing agent in tissue processing. It is an excellent lipid solvent but has the negative characteristic of drying tissue specimens. Xylene is "practically insoluble" in water and therefore is capable of removing water from the tissue, especially in the longer clearing times needed for fatty tissue. Properly dehydrated tissue at this point in processing has only bound water and the removal of this causes the excessive drying (Feldman et al., 2014). As well as its negative drying action on tissue, the health hazards of xylene are well documented (Kilburn et al., 1985; Uchida et al., 1993; Laine et al., 1993).

Alternatives to xylene and the earlier clearing agents exist which are less hazardous to health. The most common of these "xylene substitutes" are citrus-based clearants and aliphatic hydrocarbons. The citrus-based clearants (Clearene, Hemo-De®, Histo-Clear®, MasterClear™, Histo-SolveX™, Ameri-Clear®, Citri-Solve™, Citra-Clear, Citrus Clearing Solvent) are made from d-limonene. D-limonene has a characteristic citrus odor, thus the name "citrus-based clearants", and an oily consistency. The degree of oiliness and smell is dependent on whether the d-limonene is diluted with other solvents. Some limonene clearants are marketed as GRAS (Generally Regarded As Safe), a Food and Drug Administration (FDA) term used to designate that a chemical is

approved as a food additive, but this classification is inappropriate for use with a clearant. D-limonene is a skin sensitizer and irritant.

Aliphatic (straight chain) hydrocarbons are not soluble in water and because they cannot remove bound water they do not dry out the tissues. Most aliphatic hydrocarbons are practically odorless, non-sensitizing and non-irritating to the skin. The degree of oiliness and flammability, if any, is dependent on the carbon chain length. Thus, the physical characteristics of proprietary aliphatic hydrocarbon blends (Clear-Rite™ 3, Pro-Par Clearant, Safe-Clear, Shandon™ Xylene Substitute, SlideBright™, Clarify™, Formula 83, Neo-Clear®, Sub-X Clearing Medium, XS-3™ Xylene Substitute) vary based on the carbon chain length and/or additives.

Infiltration

Paraffin wax

After clearing, tissue sections are infiltrated (impregnated) with paraffin wax to support the tissue, allowing thin sections to be cut. Infiltration must be sufficient to displace the clearant from the tissues, otherwise the wax will not harden properly and it interferes with microtomy. Tissues which are not allowed to infiltrate for long enough will be soft and crumbly making sectioning difficult. Too much time in high temperature wax can cause excessive shrinkage and produce dry, brittle tissues which are equally difficult to section. Proprietary formulations utilize various paraffin waxes and polymers to assist in the successful microtomy of diverse tissue types and section thicknesses. Wax selection should be based on the density of the tissue it must support. Low melting point wax typically has higher paraffin concentrations and will provide a softer matrix. Waxes with higher polymer content produce a harder matrix which will mimic dense tissue more closely. Once infiltration is complete, tissues are embedded into a paraffin wax block to enable sectioning on a microtome. The temperature of the embedding paraffin wax should be 2-4° above its melting point. Overheating of the wax can cause hardening and distortion of the tissue and even a breakdown of the paraffinic additive (e.g. resin polymer) resulting in poor performance. Paraffin wax is inexpensive, provides quality sections and is easily adaptable to a variety of uses. It is also compatible with most routine and special stains including immunohistochemistry protocols.

Alternative infiltration media

Occasions when paraffin wax is an unsuitable medium include:

- Processing reagents remove or destroy tissue components which are the object of investigation, e.g. lipids.
- The use of heat may have an adverse effect on a tissue component, e.g. enzymes.
- Sections are required to be thinner, e.g. electron microscopy.
- The infiltrating medium is not sufficiently hard to support the tissue, e.g. undecalcified bone.

Resin

This is used exclusively as the embedding medium for electron microscopy (see Chapter 21), ultra-thin sectioning for high resolution and undecalcified bone (see Chapter 17).

Agar

Agar gel alone does not provide sufficient support for sectioning tissues. Its main use is as a cohesive agent for small friable pieces of tissue after fixation, a process known as double embedding, when fragments of tissue are embedded in melted agar, allowed to solidify and trimmed for routine processing. Another method is to filter the fixative containing small, friable tissue fragments through a Millipore filter using suction. Molten agar is then carefully poured into the filter apparatus, the agar is left to solidify and the resultant agar pellet is removed and routinely processed and embedded in paraffin wax. An equivalent system is the use of cheese spread (see Chapter 5).

Gelatin

This is primarily used in the production of sections of whole organs using a modification of the Gough-Wentworth technique (Whimster, 1969),

in combination with agar as a pre-embedded medium and in frozen sectioning.

Celloidin

Celloidin is used when processing dense and/or hard tissues. The use of celloidin or low viscosity nitrocellulose (LVN) requires special equipment and non-conventional microtomy techniques and is not suitable for clinical histology laboratories.

Embedding (Blocking)

This is the process of creating tissue blocks by using an external support medium to enable microtomy. The embedding medium should be completely compatible with the infiltrating medium in order to prevent tissue section separation and to facilitate sectioning.

Paraffin wax embedding

Most laboratories use modular embedding centers which consist of a paraffin wax dispenser, cold plate and heated storage area for molds and processed tissue cassettes. Paraffin wax is dispensed from a nozzle into a suitably sized, warm mold. The tissue is oriented in the mold, fixed to the bottom of it using the cold plate and then a cassette is placed on top of the mold and completely filled with wax. This final tissue block is placed on a cold plate to allow the paraffin wax to solidify.

Resin embedding

This process is similar to paraffin wax embedding, however these blocks are hardened (polymerized) with heat, ultraviolet light or chemical catalysts, depending on the type of resin used (see Chapter 8).

Tissue orientation

The ability to see the desired tissue morphology in the section is dependent on the correct placement or orientation of the sample in the block. Incorrect orientation may damage diagnostic tissue elements during microtomy or obscure them from microscopic view, preventing a correct diagnosis. Products are available which assist with proper orientation, e.g. marking systems, tattoo dyes, biopsy bags, sponges and papers. Each laboratory should make use of these indicators to alert the embedder appropriately, e.g. a piece of green paper, meaning embed on edge, or instructions etched directly onto the cassette. Orientation of the tissue should offer the least resistance of the tissue against the knife during sectioning, e.g. long tissue samples should be placed diagonally in the block rather than straight across as a margin of embedding medium around the tissue provides additional tissue support.

Tissues requiring special orientation include:

- Tubular structures, e.g. arteries, veins, fallopian tube and vas deferens: cross sections of the wall and lumen should be visible.
- Skin biopsies: embed shave biopsies on edge and punch biopsies on their side so that all the epidermis, dermis and hypodermis are visible.
- Nails: embed on edge.
- Intestine, gallbladder and other epithelial tissue: orient so the epithelial surface is cut last, minimizing compression and distortion of this layer.
- Multiple tissue pieces: place side by side with the epithelial surfaces facing in the same direction.

Tissue processors

The open, tissue transfer processors were the first automation to replace hand processing in the histology laboratory. Open processors transport tissue cassette loaded baskets through stationary containers of processing reagents. Early models used a notched clock disk to electronically control the time, and agitation occurred by mechanically raising and lowering the baskets within each reagent container. The circular configuration of the processor, and the up and down agitation gave them their "carousel model" nickname. Carousel models offered a great improvement over hand processing and led the way for machines which could provide even greater efficiency and safety.

Self-contained, closed, fluid transfer processors quickly followed and continue to be the most

popular instrument in histology laboratories today. A microprocessor-based control is used to program the instrument and multiple programs can be created and saved. The processing reagents are pumped sequentially in and out of the retort chamber where the tissue cassettes are loaded, greatly reducing exposure to solvent vapors. Processing time, temperature, and/or pressure/vacuum (P/V) can be customized for each solvent station. This offers the advantage of creating customized schedules for different tissue sizes and types, e.g. standard (overnight) versus biopsy runs (two hours). These processors employ alarm systems and diagnostic programs for troubleshooting any instrument malfunction. Newer instruments have divided retorts which allow different programs to run simultaneously, improving utilization of the equipment and providing the opportunity to divide tissue by size on the same run. Many processors have solution management systems which monitor the solution concentrations to indicate when a change is required (various processor manuals, see useful websites and other useful information).

Microwave processors

Enclosed tissue processors have the option of adding heat to the chamber to warm the solvent from any station, this accelerates diffusion into the tissue at a faster rate than at room temperature. Microwaves however, which are present on newer processor models, generate heat almost instantaneously and this is a much faster process than heat conduction alone (Giberson, 2001; Kok & Boon, 2003). The longer tissue is exposed to heat the greater the chance of producing heat artifacts, e.g. irregular staining patterns, excessive hematoxylin staining and dry tissues.

Microwave tissue processors are not equivalent to standard kitchen models with on-off cycles which vary in length depending on cooking times and power settings. Microwave processors use shorter cycle times which are consistent regardless of the programmed time. This correct use of microwaves during tissue processing can shorten processing times without producing heat artifacts.

Microwave tissue processor models range from those which require manual placement of reagents into the chamber to those which are completely automated. Proprietary solvents and schedules are available to work optimally with the microwaves, or schedules are modified to work with conventional processing solvents. If manual microwave processing is being conducted, great care should be taken to prevent exposure to the heated vapors. Microwaves have penetration limits so specimens should not be grossed thicker than 3 mm, as thicker specimens would require the same time as conventional processing.

Advances in technology have led to the development of a 'continuous input rapid tissue processor'. This enclosed processor uses microwave technology, vacuum infiltration and proprietary reagents which are described as being 'molecular-friendly'. A robotic arm moves the tissue cassettes through the stations which contain acetone, isopropanol, polyethylene glycol, mineral oil and paraffin wax. Microwaves and agitation are used to accelerate the diffusion of solvents into the tissue. A patented microwave technology is utilized which operates at a continuous low power instead of pulsing high levels of microwave energy. The cylindrical retort chamber allows microwaves to circle around the cavity and eliminates hot and cold spots. Continuous throughput allows loading of tissues into the system every 20 minutes, improving workflow through the laboratory and subsequent turnaround times.

Advantages of new technology in processing

- Custom programs specific to tissues being processed, addition of vacuum/pressure, agitation or heat at any stage.
- Rapid schedules.
- Fluid and fume containment.
- Time delay for start of processing schedules.
- Reagent management and volume reduction.

Processor maintenance

Every laboratory should have a policy outlining the rotation and changing of solutions on the tissue

processor. The numbers, sizes and types of tissue processed, and the reagents used will play a role in the determination of this policy. Solutions should be carefully monitored to ensure quality. Every manufacturer has a handbook outlining an appropriate maintenance schedule.

Important maintenance tips

- Any spillage or overflow should be cleaned immediately.
- Accumulation of wax on any surface should be removed.
- Paraffin wax bath temperatures should be monitored daily.
- Warm water (or manufacturer recommended solution) flushes should be performed regularly to keep the lines free of salts, protein and debris.

Processing schedules

Manual tissue processing has stopped in most laboratories. However, certain circumstances, i.e. a power failure or equipment malfunction, may necessitate its reintroduction.

Although rapid processing schedules for small biopsies or urgent specimens have become increasingly mainstream, overnight processing is still considered to be the routine schedule in many laboratories.

Tissues typically start in a fixative station, where they are placed in 10% phosphate buffered formalin, to assure complete fixation. The fixative is also the best "hold" solution for specimens on a delayed start program. The process may include an alcoholic fixative, incrementing concentrations of alcohol, xylene (or xylene substitute) followed by infiltration in paraffin wax. Schedules are customized for the tissues being processed. Factors influencing the processing schedule include end time required, reagents used, the inclusion of heat and vacuum, and the size and number of tissue cassettes processed. It has been found that successful processing schedules, whether they are short or long, have proportionally equal times in the major processing steps of dehydration, clearing and infiltration. The ratio for timing in routine processing schedules is 40% dehydration, 30% clearing and 30% infiltration. For xylene-free processing, the ratio is 35% dehydration (alcohols, alcohol mixtures), 30% clearing (isopropanol), and 35% infiltration (wax) (Rolls, 2014).

Table 6.1 Processing schedule #1

Processor: Tissue-Tek® VIP® 5, vacuum, no heat except wax baths

Time: Overnight, 14 hours

Tissue: General surgical specimens, 3 mm thick, ≤ 20 x 20 mm

Station	Reagent	Time
1	NBF	1 hour + hold time
2	NBF	45 min
3	70% reagent alcohol	45 min
4	80% reagent alcohol	45 min
5	95% reagent alcohol	45 min
6	100% reagent alcohol	45 min
7	100% reagent alcohol	45 min
8	Pro-Par Clearant	45 min
9	Pro-Par Clearant	45 min
10	Pro-Par Clearant	45 min
11	Paraffin wax	30 min
12	Paraffin wax	30 min
13	Paraffin wax	30 min
14	Paraffin wax	30 min

There are no "standard" processing schedules although manufacturers do have their own programs designed to run specifically on their processor. Differences in the tissue types and size, number of cassettes, required turnaround time, processor models and choice of reagents all play a major role in determining an optimal processing schedule. (Tables 6.1-6.6).

Special considerations

Prognostic and predictive markers

Histology has always played an important role in diagnosis and subsequent treatment options. The

Table 6.2 Processing schedule #2 (personal communication Pam Marcum)

Processor: Excelsior, no vacuum, agitation and no heat except wax baths

Time: Overnight, 14 hours

Tissue: General surgical specimens

Station	Reagent	Time
1	NBF	45 min + hold time
2	NBF	1 hour
3	75% reagent alcohol	45 min
4	85% reagent alcohol	45 min
5	3rd reagent alcohol	45 min
6	4th reagent alcohol	45 min
7	5th reagent alcohol	45 min
8	6th reagent alcohol	45 min
9	7th reagent alcohol	45 min
10	8th reagent alcohol	45 min
11	Xylene	45 min
12	Xylene	1 hour
13	Paraffin wax	45 min
14	Paraffin wax	45 min

Note: Processor starts with 100% reagent alcohol in stations 5-10. The percentage of the alcohols is monitored by the processor and rotated based upon set criteria.

Table 6.3 Processing schedule #3 (personal communication Pam Marcum)

Processor: Leica ASP 300s, no vacuum, no heat except wax baths

Time: 4 hours

Tissue: Bone marrow

Station	Reagent	Time
1	NBF	15 min
2	NBF	15 min
3	70% alcohol	15 min
4	95% alcohol	15 min
5	95% alcohol	15 min
6	100% alcohol	15 min
7	100% alcohol	15 min
8	100% alcohol	15 min
9	Xylene	15 min
10	Shandon™ Xylene Substitute	20 min
11	Shandon™ Xylene Substitute	20 min
12	Paraffin wax	10 min
13	Paraffin wax	10 min
14	Paraffin wax	10 min

advent of molecular testing can evaluate a patient's overall outcome (prognosis) or identify whether a patient qualifies for a specific therapy (predictive), making it imperative to produce the highest quality tissues.

Standardization of the fixation and processing of tissue has become a focus due to the increased use of companion diagnostics. Pre-analytic, analytic and post-analytic variables are addressed in the published guidelines for tests linked to companion drug treatments. The requirements given ensure accurate demonstration of prognostic markers.

The pressures to comply with the new standards and guidelines is forcing histology laboratories to revisit their processing schedules and adapt or adjust protocols accordingly.

Restoration of tissue dried in processing

Despite precautions taken during processing, technical or mechanical malfunctions and human error may occur, resulting in tissue drying out prior to paraffin wax impregnation. Tissue restoration can be accomplished by placing the tissue in a formol-glycerol solution for 5-10 hours (Ellis, 2016). Whilst the tissue may not be ideal, this may provide slides of adequate diagnostic quality. Processing begins with the dehydrating solutions and continues to completion. The tissue may be

Table 6.4 Processing schedule #4

Processor: Tissue-Tek® VIP® 5, vacuum, no heat except wax baths

Time: Overnight, 14 hours

Tissue: General surgical specimens, 3 mm thick, ≤ 20 x 20 mm

Station	Reagent	Time
1	NBF	1 hour 15 min + hold time
2	70% reagent alcohol	30 min
3	95% reagent alcohol	30 min
4	99% reagent alcohol	30 min
5	99% reagent alcohol	60 min
6	99% reagent alcohol	60 min
7	99% reagent alcohol	60 min
8	Xylene	60 min
9	Xylene	1 hour 15 min
10	Xylene	1 hour 15 min
11	Paraffin wax	60 min
12	Paraffin wax	1 hour 15 min
13	Paraffin wax	1 hour 15 min
14	Paraffin wax	1 hour 15 min

Table 6.5 Processing schedule #5

Processor: Leica Peloris, vacuum, no heat except wax baths

Time: Rapid, 3 hours 15 min

Tissue: Unfixed core biopsies, small tissue and endoscopic biopsies

Station	Reagent	Time
1	NBF	1 hour
2	70% IMS*	10 min
3	95% IMS	10 min
4	99% IMS	10 min
5	99% IMS	10 min
6	99% IMS	10 min
7	99% IMS	10 min
8	Xylene	10 min
9	Xylene	10 min
10	Xylene	10 min
11	Paraffin wax	10 min
12	Paraffin wax	10 min
13	Paraffin wax	10 min
14	Paraffin wax	15 min

*IMS, industrial methylated spirit.

difficult to section and coated or adhesive slides should be used.

Reprocessing of poorly processed paraffin wax-infiltrated specimens

Melt down excess wax and reprocess normally, beginning in fixative. Correctly processed tissue will be protected from over-processing by the residual wax whilst under-processed areas remain exposed and will have a second chance at dehydration.

Quality control

Temperature of all paraffin wax dispensers, flotation water baths and automated processors are carefully monitored, maintained and documented. A hydrometer can be used to measure the specific gravity (relative density) of the alcohols. This reading is useful in determining rotation schedules. The histology laboratory should have a policy and procedure manual which addresses quality issues and corrective actions to be taken.

Summary

Technological advances have been made in tissue processor instrumentation, partly due to increased workload, the demand for a faster turnaround time for diagnostic samples and the reduction in workforce. The addition of microprocessors, microwaves, and environmentally friendly chemicals are only a few of the improvements which continue to advance tissue processing.

Table 6.6 Processing schedule #6

Processor: Tissue-Tek® VIP® 5, vacuum, no heat except wax baths

Time: 64 hours

Tissue: Specifically brain blocks

Station	Reagent	Time
1	95% IMS	5 hours
2	99% IMS	5 hours
3	99% alcohol	5 hours
4	99% alcohol	5 hours
5	99% alcohol	5 hours
6	Isopropyl alcohol	5 hours
7	Isopropyl alcohol	5 hours
8	Xylene	4 hours
9	Xylene	4 hours
10	Xylene	5 hours
11	Paraffin wax	4 hours
12	Paraffin wax	4 hours
13	Paraffin wax	4 hours
14	Paraffin wax	4 hours

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7 Microtomy for paraffin and frozen sections

Lena T. Spencer

Introduction

Microtomy is the means by which tissue is sectioned and attached to the surface of a glass slide for further microscopic examination. The basic principles are applicable to both paraffin and frozen sections although most microtomy is performed on paraffin wax-embedded tissue blocks.

The instrument used to cut sections is the microtome. This has an advancing mechanism which moves the object, the paraffin block, for a predetermined distance until it is in contact with the cutting tool, the knife or blade. The specimen moves vertically past this cutting surface and a tissue section is produced.

This chapter will discuss the techniques necessary to provide quality slides for microscopy in clinical and research histology. Good technique takes practice.

Types of microtome

There are several types of microtome, each designed for a specific purpose, although many have multifunctional roles.

Rotary microtome

This is often referred to as the "Minot" after its inventor. The basic mechanism requires the rotation of a fine advance hand-wheel by 360° degrees, moving the specimen vertically past the cutting surface and returning it to the starting position. The rotary microtome may be manual, i.e. completely manipulated by the operator; semi-automated with one motor to advance either the fine or coarse hand-wheel; or fully automated, when two motors drive the fine and the coarse advance hand-wheel. The mechanism for block advancement may be retracting or non-retracting. Its advantages include the ability to cut thin 2–3 µm sections and its easy adaption to all types of tissue sectioning. Technological advances in the automation of microtomy have improved section quality, increased productivity and improved occupational safety for the technologist. Eliminating manual hand-wheel operation of the microtome reduces the incidence of repetitive motion disorders, a common occupational health problem in the histology laboratory.

Base sledge microtome

Here the specimen is held stationary and the knife slides across the top of it during sectioning. Used primarily for large blocks, hard tissues or whole mounts, it is especially useful in neurological and ophthalmic pathology but 3 µm sections are difficult to produce. Further information regarding sectioning of undecalcifed bone is available in Chapter 17.

Rotary rocking microtome

Commonly used in cryostats, the retracting action moves the tissue block away from the knife on the upstroke, producing a flat face to the tissue block.

Sliding microtome

The knife or blade is stationary and the specimen slides under it during sectioning. This microtome was developed for use with celloidin-embedded tissue blocks used primarily for research.

Ultra-microtome

Used exclusively for electron microscopy, see Chapter 21.

Microtome knives

There are many shapes, sizes and materials for microtome knives. Knives were developed to fit specific types of microtome and to cope with different degrees of hardness of tissues and embedding media. Most steel knives have been replaced with disposable blades, although exceptions include the tool-edge knives for resin and steel knives for some cryostats.

Disposable blades

These have revolutionized microtomy. Disposable blades are used for routine microtomy and cryotomy, providing a sharp cutting edge which can produce almost flawless 2–4 µm sections. Disposable blade holders are incorporated into the microtome or an adaptor may be purchased. The blades may be purchased in dispensers, with or without a polytetrafluoroethylene (PTFE) coating which allows ribbons to be sectioned with ease. Note:

- The clearance angle should be adjusted in small increments to eliminate problems which occur with ribboning of the tissue. Low profile blades have a clearance angle of 3-5º, high profile 5-7º.
- Over-tightening the disposable blade in the clamping device may cause cutting artifacts, e.g. thick and thin sections.
- The clamping device must be clean and free of defects.
- During sectioning the hand-wheel must be turned slowly.
- Extremely hard tissues may pose a problem for disposable blades.

Reliability of a constant sharp edge, ease of use, low or high profiles adaptable to a variety of tissue and paraffin types, and low cost relative to steel knife sharpening, make these blades the mainstay in most laboratories.

Glass and diamond knives

Glass and diamond knives are used in electron microscopy and with plastic resin-embedded blocks.

Paraffin section cutting

Equipment required

- Flotation (water) bath.
- Slide drying oven or hot plate.
- Fine pointed or curved forceps.
- Sable or camel haired brush.
- Scalpel.
- Slide rack.
- Clean slides.
- Teasing needle.
- Ice tray or cooling platform.
- Chemical-resistant pencil or pen.
- Electronic slide labeling instrument.

Flotation (water) bath

A thermostatically controlled water bath is used for floating out tissue ribbons after sectioning. The temperature of the water in the bath should be 10°C below the melting point of the paraffin wax to be sectioned. Care should be taken to prevent water bubbles from being trapped under the section and this can be accomplished by using distilled water in the bath. Alcohol or a small drop of detergent may be added to the water to reduce the surface tension allowing the section to flatten out with ease.

Drying oven or hot plate

Drying ovens incorporate fans which keep the warm air circulating around the slides. The temperature setting should be approximately that of the melting point of the paraffin wax. If the oven is too hot there may be distortion to the cells causing dark pyknotic nuclei, nuclear bubbling and loss of nuclear detail. Drying times vary depending on the type of tissue, the number of slides to be dried and size of the drying device. Many automated stainers have drying ovens as part of the instrument and the time and temperature is easily regulated. Special care should be taken when drying delicate or central nervous system tissue, a lower temperature is required to prevent splitting and cracking of the section and 37°C for 24 hours is recommended. Time for drying slides should be monitored as many immunohistochemical or special stains are heat sensitive.

Brush and forceps

These or teasing needles are helpful in removing folds, creases and bubbles which may form during floating out of the section on the water bath. They are also helpful for manipulating the section as it passes across the edge of the blade.

Slides

For normal routine work, 76 × 25 mm slides are universally used. Although slides are available in a variety of thicknesses, 1.0–1.2 mm thickness are preferred because they do not break easily. Most slide racks are made to accommodate this slide size. Larger slides are available for use with specialty tissues such as eyes or brains. Unique identification numbers or codes, patient name or other information should be etched, embossed or written on each slide. Automated instruments which imprint the patient's information on the glass slide are available. Chemical-resistant pens and pencils are routinely used to label the slide. Slides which are positively charged or pre-treated with an adhesive resist detachment of the tissue from the slide during staining. Colored, frost-ended slides may be used for specialized techniques.

Section adhesives

Providing clean slides are used and sections are adequately dried, the problem of sections detaching from the slide during staining should not occur. Occasions when sections may detach from the slide are:

- Exposure to strong alkali solutions during staining.
- Cryostat sections for immunofluorescence, immunohistochemistry or intraoperative consultation.
- Central nervous system (CNS) tissues.
- Sections which are submitted to extreme temperatures.
- Tissues containing blood and mucus.
- Decalcified tissues.

Adhesives may alleviate the problem of tissue loss. Protein adhesives such as albumen, gelatin and starch may be prone to bacterial growth or heavy staining but close monitoring will prevent these problems. Other adhesives which may be used are:

Poly-L-lysine (PLL)

This is bought as a 0.1% solution which is further diluted for use 1:10 with distilled water. Slides are coated with the diluted solution and allowed to dry. The effectiveness of the coating to adhere the tissue to the slide will diminish within a few days.

3-aminopropyltriethoxysilane (APES)

Slides are dipped in a 2% solution of APES in acetone, drained, dipped in acetone and drained again. The process is complete when the slides are dipped in distilled water and placed upright in a rack to dry. These slides are useful for cytology and specimens which may be bloody or contain proteinaceous material.

Charged or plus slides

Laboratories often use slides which have been manufactured with a permanent positive charge. Placing a positive charge on the slides is accomplished by coating the slide with a basic polymer in which a chemical reaction occurs, leaving the amino groups linked by covalent bonds to the silicon atoms of the glass. These slides are superior in their resistance to cell and tissue loss during staining or pre-treatments such as enzyme and antigen retrieval.

Practical microtomy

The expertise which must be gained to become a competent microtomist cannot be achieved from textbooks. Practical experience under the guidance of a skilled tutor is the best way to gain the confidence and coordination necessary to manipulate the microtome and the sections produced. Techniques will be described providing information and helpful hints for use during microscopy.

Setup of the microtome

Maintenance of the microtome is important to the production of quality slides for diagnosis. The manufacturer's recommendation regarding the proper care of the instrument should be closely followed. A departmental policy should be implemented

outlining daily, weekly, quarterly and yearly preventive maintenance procedures.

The water bath and the microtome should be ergonomically positioned to reduce stress and tension on the employee's neck and shoulders. The water bath may be filled with distilled or tap water and adjusted to the correct temperature for the paraffin wax. Care should be taken to reduce air bubbles which may distort the tissue section. The temperature of the water bath should be recorded and monitored throughout the day for quality purposes.

The blade should be sharp and defect free. The blade or knife holder should be adjusted to optimize the clearance angle, the distance between the lower facet angle and the surface of the block face. The recommended angle varies from 2–4° for paraffin and 5–7° for frozen sections. The correct angle reduces friction as the blade passes through the block, preventing compression of the section. Determining the exact angle is largely a matter of trial and error. Clamps and screws must be firmly tightened. If a disposable blade is to be used, care should be taken to ensure enough pressure is being exerted on the blade to provide support, but it should not be over-tightened, as this causes thick and thin sectioning.

Sectioning

Trimming the tissue blocks

The paraffin block may be faced or "rough cut" by setting the micrometer at 15–30 µm or by advancing the block using the coarse feed mechanism. Aggressive trimming will cause "moth hole" artifacts. Care must be taken to ensure that the block clamped in the chuck has been retracted so that there is no contact with the blade on the initial downstroke. It is possible to damage the tissue by gouging or scoring when trimming the block.

Cutting sections

Blocks should be arranged in numerical order on an ice tray or cooling mechanism, cooling both the tissue and the paraffin wax to a consistent temperature. A small amount of water is absorbed into the tissue causing slight swelling and making sectioning easier. Over-soaking may cause expansion and distortion of the tissue section. Proper processing greatly reduces or eliminates the need to pre-soak blocks. Routine surgical material should be cut at 3–4 µm. The micrometer setting does not guarantee that each section will be that exact thickness. Thickness depends on many factors including temperature, knife angle and cutting speed. Experience will determine the speed of the stroke but in general, one should use a smooth, slow stroke. If there is difficulty cutting a smooth flat section, warming the block face with warm water, or gently exhaling breath onto the block surface during sectioning may help. This has the effect of expanding the block, giving a slightly thicker section. Ideally, successive sections will stick edge to edge, forming a ribbon. If the entire block is to be sectioned and retained, the ribbons are stored in a receptacle for future use. Ribbons are the most convenient way of handling sections. When a ribbon of several sections has been cut, the first section is held by forceps or teasing needle and the last section eased from the knife edge with a small brush.

Floating out sections

The floating out of the ribbon must be smooth, the trailing end of the ribbon making contact with the water first. The slight drag produced when the rest of the ribbon is laid on the water is sufficient to remove most, if not all of the folds which occur. Sections are floated on the water bath shiny side down. Folds in the section may be removed by simply teasing with the forceps. Approximately 30 seconds should be long enough for a ribbon to flatten, longer on the water causes excessive expansion distorting the tissue. Individual sections or ribbons may be floated onto the slide.

Circular structures such as eyes may be difficult to flatten. Various techniques are useful in these situations, e.g. placing the section on a slide which has been pre-flooded with 50% alcohol. The slide is gently immersed in the water bath and the section of eye will float on the surface. The presence of the alcohol will set up diffusion currents which help flatten the tissue section.

The water bath should be cleaned after each block is cut, removing debris and tissue fragments by dragging tissue paper across the surface. Cleanliness

cannot be overemphasized as debris from different sections (carryover) is a serious problem.

Drying sections

The small amount of water held under the section will allow further flattening to occur when heat is applied to dry the section. The temperature should be at the melting point of the paraffin wax. Automated stainers have integral drying ovens. Slides may be attached to the stainer with individual slide holders or in racks which are designed for the instrument. It is important to eliminate over-heating during the drying stage as cellular details may be compromised. Hot plates may cause localized overheating of the drying delicate tissues; less distortion will occur if the temperature is reduced and the time prolonged. Overnight drying at 37°C or room temperature is recommended for many tissues.

Cutting hard tissues

This has become less problematic since the introduction of disposable blades. Cutting difficulties are more likely due to poor fixation or over-processing. Prolonged soaking of the block or exposing the block surface to running tap water for 30 minutes overcomes many of the problems associated with cutting hard tissues. A slight reduction in the knife angle may also yield results. If these remedies fail, softening agents may be used on the surface of the block.

Surface decalcification

When small foci of calcium are present in the tissue section, cutting a quality section may be difficult. The block may be removed from the chuck after rough cutting the tissue and placed face down in a dish which contains a small amount of decalcifying solution. The exposure time will vary depending on the tissue and requires close monitoring. The block is rinsed well, blotted dry, chilled and returned to the microtome. An immediate section should be taken since the decalcification achieved will be limited. Diagnostic materials may be compromised if over-decalcification occurs. It must be noted that the staining properties of the tissue may be affected after this treatment and allowances must be made to achieve optimum results.

Problems and solutions

Table 7.1 addresses the most common problems encountered during microtomy and their possible solutions.

Frozen and related sections

This is a discussion of the methods used to produce sections which preserve cellular morphology without the use of dehydrating and clearing solutions and heat. Frozen sections have important clinical and research applications. Clinically, frozen sections are used for rapid intraoperative diagnoses, Moh's procedures for surgical margins and sentinel node evaluation. All of which have great significance in patient management. The technical skills needed to produce quality, interpretable slides require an understanding of microtomy and the anatomy of the tissue being sectioned. Good hand-eye coordination, attention to detail and the ability to work under pressure are essential traits needed to perform this task correctly.

Uses of frozen sections

Frozen sections have many applications in histology laboratories including:

- Intraoperative diagnosis.
- Diagnostic and research enzyme histochemistry for labile enzymes.
- Immunofluorescence.
- Immunohistochemistry techniques when heat and fixation may inactivate or destroy the antigens.
- Diagnostic and research non-enzyme histochemistry, e.g. lipids and some carbohydrates.
- Silver stains, particularly in neuropathology.

Theoretical considerations

The principle of cutting frozen sections is simple: when the tissue is frozen, the interstitial water in the tissue turns to ice and in this state the tissue is firm, the ice acting as the embedding medium. The consistency of the frozen block may be altered by varying the temperature of the tissue. Reducing the temperature will produce a harder block; raising the temperature makes the tissue block softer. The majority of non-fatty unfixed tissues section well at −20°C. The sectioning of fixed tissue requires a block

Table 7.1 Problems and solutions for paraffin wax sectioning

Causes	Solutions
Ribbon of consecutive sections are curved	
Block edges not parallel	Trim block until parallel
Dull blade edge	Replace blade or move to a different area
Excessive paraffin wax	Trim away excess paraffin wax
Tissue varying in consistency	Re-orient block
Thick and thin sections	
Paraffin wax too soft for tissue or conditions	Cool block with ice or re-embed in higher melting point wax
Insufficient clearance angle	Increase clearance angle
Faulty microtome mechanisms	Maintain microtome – lubricate and calibrate. Check for obvious faults with microtome, parts may be worn
Blade or block loose in holders	Tighten block and blade
Chatter – thick and thin zones parallel to blade edge	
Blade or block loose in their holders	Tighten blade and block holders
Excessively steep clearance angle or knife tilt	Reduce angle
Tissue or paraffin wax too hard for sectioning	Use softening fluid
Calcified areas in tissue	Rehydrate and surface decalcify
Over-dehydration of the tissue	Re-embed in fresh paraffin wax
Dull blade	Replace or use new area of blade, clean blade edge to remove excess paraffin wax
Splitting of sections at right angles to knife edge	
Nicks in blade	Use different part of blade or replace
Hard particles in tissue	Surface decalcify if calcium deposit
Hard particles in paraffin wax	Remove with fine sharp pointed scalpel if mineral or other particle
Sections will not form ribbons	
Paraffin wax too hard for sectioning conditions	Re-embed in lower melting point paraffin wax
Debris on knife edge	Clean with xylene moist cloth
Clearance angle incorrect	Adjust to optimal angle
Sections attach to block on return stroke	
Insufficient clearance angle	Increase clearance angle
Debris on blade edge	Clean with xylene moist cloth
Debris on block	Trim edges of block
Static electricity on ribbon	Humidify the air around the microtome, place static guard or dryer sheets near microtome
Incomplete section	
Incomplete impregnation of the tissue with paraffin wax	Reprocess tissue block
Tissue incorrectly embedded	Re-embed tissue, make sure orientation is correct and the tissue is flat in mold
Sections superficially cut	Re-face block, cut deeper into tissue
Excessive compression	
Dull blade	Replace blade
Paraffin wax too soft for the tissue	Cool block face and re-cut

Continued

Table 7.1 Problems and solutions for paraffin wax sectioning—cont'd	
Causes	**Solutions**
Sections expand or disintegrate on water bath	
Poor impregnation of tissue	Re-process tissue
Water temperature too high in flotation bath	Reduce the temperature of the flotation bath
Sections roll into a coil instead of remaining flat on knife edge	
Blade dull	Use new blade
Rake angle too small	Reduce blade tilt if clearance angle is excessive
Section too thick	Reduce section thickness

temperature of approximately –10°C or warmer. There is more water in fixed tissue so the tissue has a harder consistency, requiring a higher temperature to obtain the ideal consistency for sectioning.

The cryostat

This is a refrigerated cabinet in which a modified microtome is housed. All the controls for the microtome are operated outside the cabinet. The first cryostats were introduced in 1954 and the following developments in design have improved both sectioning and laboratory safety:

- Electronic temperature control.
- Electronically controlled advance and retraction of the block.
- Specimen orientation facility.
- Digital visualization of chuck and cabinet temperature.
- Mechanical control of cutting speed and section thickness.
- Automatic defrost mechanism.
- Automated decontamination and sterilization.
- Freezing shelf / bar or a "Peltier" device.

Freezing of fresh unfixed tissue

The fresh tissue should be frozen as rapidly as possible without creating freeze artifacts. Suitable techniques include:

- Liquefied nitrogen (–190°C).
- Isopentane (2-methylbutane) cooled by liquid nitrogen (–150°C).
- Dry ice (–70°C).
- Carbon dioxide gas (–70°C).
- Aerosol sprays (–50°C).
- Internal freezing shelf or bar.

Freeze artifact occurs when the water in the tissue freezes and forms ice crystals; the size and quantity of crystals is proportional to the speed at which the tissue is frozen. The tissue is cut and the sections placed on slides at room temperature; at this point the tissue section is thawed. The thawing of the ice crystals produces freeze artifact which appears as holes, or a discontinuation of the tissue architecture when viewed microscopically.

The best frozen sections are obtained when the tissue is frozen quickly. The method of choice is isopentane cooled by liquid nitrogen. The problem with using liquid nitrogen alone is the formation of nitrogen vapor bubbles around the tissue which act as an insulator and inhibit rapid, even cooling of the tissue. This can produce freeze artifact in the tissue making diagnostic interpretation difficult, especially in muscle biopsies. This problem can be overcome by snap freezing the tissue in an agent with a high thermal conductivity which has been cooled to approximately –160°C by immersion of the isopentane in liquid nitrogen. A beaker of isopentane is suspended in a flask of liquid nitrogen. When the temperature of the isopentane reaches –160°C, the tissue affixed to a cork disk, aluminum foil or a cryostat chuck is submerged in the isopentane. Insufficient time in the freezing medium can lead to freeze artifact, whilst prolonged freezing may crack

the block compromising the sample and causing sectioning problems. The tissue may be rolled in talc prior to snap freezing to reduce freezing artifact.

Solid carbon dioxide (dry ice) may be used for freezing tissue blocks. Two pieces of dry ice are held in gloved hands against the cryostat block holder containing the tissue which has been oriented in a cryoembedding medium. As the tissue freezes, a white line will be seen passing through it. The dry ice should then be removed to avoid over-freezing. This method is not economical since the dry ice will return to the gaseous state upon storage. The need for regular deliveries and the wastage of large amounts of dry ice are disadvantages to this method.

Carbon dioxide gas from a CO_2 cylinder has been successful in the past. Tissue blocks are frozen by adapting a conventional freezing microtome with a gas supply or by using a special adaptor for the CO_2 tank which holds the tissue chuck.

Aerosol sprays have gained popularity as a means of freezing small tissue blocks. These sprays have the advantage of being readily available and easily stored. A major problem is the environmental issues of aerosol emissions. Additionally there is a risk to the scientist of both aerosol inhalation and possible contamination by microbial exposure released from the tissue by the spray.

Tissue can be frozen directly in the cryostat using the freeze bar and the heat extractor. Newer cryostats are equipped with a "Peltier" device, a thermoelectric cooling mechanism which increases the diffusion of heat from the tissue, producing rapid freezing. The tissue is frozen simultaneously from the block face and the block holder. Freeze artifact may be reduced if all objects are kept cold and ready for use. This method is quick and often used for intraoperative frozen sections.

Fixed tissue and the cryostat

For most diagnostic purposes in a routine laboratory, cryostat sections of unfixed tissue are suitable. However, freezing unfixed tissue causes the diffusion of labile substances. This is enhanced when the section is cut in the cryostat, producing heat which causes slight thawing of the cut section. This may not cause a problem for diagnosis, but it can affect the accurate localization of some abundant enzymes, e.g. acid and alkaline phosphatases. To accurately localize these hydrolytic enzymes and other antigens it is useful to fix the tissue prior to sectioning in the cryostat. Tissue prepared in this manner must be fixed under controlled conditions; it must be absolutely fresh and placed in formal calcium at 4°C for 18 hours. The technique is outlined below.

Gum sucrose solution

Gum acacia	2 g
Sucrose	60 g
Distilled water	200 ml

Store at 4°C

Method

1. Fix fresh tissue block in formal calcium at 4°C for 18 hours.
2. Rinse in running water, or for a short time in distilled water if the tissue fragment is small or fragile, e.g. jejunal biopsy.
3. Blot dry.
4. Place tissue in the gum sucrose solution at 4°C for 18 hours or less with small fragments.
5. Blot dry.
6. Freeze tissue onto the block holder.

Following fixation, the block is frozen slowly to avoid damage caused by the rapid expansion of ice within the tissue. Freezing the block by standing it in the cryostat cabinet produces acceptable results for the majority of fixed tissue. The length of time required for this procedure limits its value as a diagnostic tool.

Cryostat sectioning

Cabinet temperature

The temperature of the microtome and the cryostat chamber should be monitored, many cryostats having digital displays of the block and cabinet temperature. Most unfixed material will section well between −15 and −23°C. Tissues containing large amounts of water will section best at the warmer temperature, and harder tissues and those which contain fat require a colder temperature. Table 7.2 gives an indication of the suggested optimal cutting temperatures for a variety of tissue types. If sections

are shattered with chatter lines this is an indication that the block is too cold. Most fixed tissues will section best within the range of –7 to –12°C, depending on the hardness of the tissue. Small blocks of undecalcified cancellous bone can be sectioned but care must be taken to remove any cortical bone fragments prior to freezing. Most cryostats have a defrost cycle which occurs daily, care must be taken to avoid leaving a specimen in the cabinet overnight. Thawing and refreezing of the sample during the defrost stage may alter the specimen and make it unsuitable for future studies.

Microtome

This should be defrosted, cleaned and oiled according to manufacturer's recommendations if cutting problems are encountered. A policy should be in place which outlines a routine maintenance schedule for each cryostat, including a section on decontamination of the instrument.

Table 7.2 Optimal tissue freezing temperatures

Tissue	–7 to –10°C	–10 to –13°C	–13 to –16°C	–16 to –20°C	–20 to –25°C	–25 to –30°C
Adrenal			X			
Bladder		X				
Bone marrow					X	
Brain	X					
Breast					X	
Breast with fat						X
Cervix				X		
Endometrium	X					
Fat					X	X
Heart				X		
Intestinal				X		
Kidney			X			
Liver	X	X				
Lung				X		
Lymphoid			X			
Muscle			X			
Ovary				X		
Pancreas				X		
Prostate				X		
Rectal			X			
Skin			X			
Spleen	X					
Testicular		X	X			
Thyroid		X				
Uterus			X			

Cryo-embedding medium

There are many different cryoembedding media commercially available. The properties of each should be carefully considered before use, including the temperature, freezing mode and type of tissue being frozen.

Blade or knife

Disposable blades have become routine in most clinical laboratories. They produce a perfect, sharp edge, are instantly available and can be rapidly cooled because of their size, but tissues which are extremely hard or dense may be troublesome. Stainless steel knives may be necessary in research and animal pathology laboratories as the type of tissue and the procedures to be performed may dictate their use. If a knife is used, sharpening techniques should be discussed in the procedure manual. A sharp edge is paramount in obtaining a quality frozen section. The microtome blade angle and block face angle should be closely monitored.

Anti-roll plate

This is attached to the front of the microtome blade adaptor and is intended to stop the natural tendency of frozen sections to curl upwards on sectioning. The device is usually made of Plexiglas or a hard plastic material. The anti-roll plate is aligned parallel to the blade edge and fractionally above it. The plate can be raised or lowered against the knife to increase the angle between the knife and the blade, the successful sectioning of a tissue block is dependent on this micro-adjustment. A new advance for the anti-roll device is the addition of a vacuum attachment which aides flattening of the section as it advances onto the blade. Anti-roll adjustments include:

- Correct height of blade edge.
- Correct angle of blade.
- Edge of plate should not be nicked or damaged.
- Cabinet temperature.

If the anti-roll plate is not working correctly, a sable hair brush can be used to manipulate the section.

Sectioning technique

Frozen sectioning requires practice to master the technique. Speed, tissue type and temperature of the block and cabinet play important roles in frozen sectioning. The cut section rests on the surface of the blade holder, a room temperature slide is held above it and electrostatic attraction causes the tissue to adhere to the slide. If tissues are being cut which require harsh or lengthy staining procedures, positively charged or coated slides should be used. These slides are usually coated with gelatin-formaldehyde (equal parts of 1% gelatin and 2% formaldehyde) or poly-L-lysine (0.01% aqueous solution). Difficult sections may also be cut and placed on the slide by use of a tape transfer system which is valuable for tissue sections which will not adhere to the slide.

Decontamination

It is extremely important for the safety of individuals using the cryostat that decontamination policies be written and followed. Many newer cryostats have disinfecting apparatus built into the cabinet using either a disinfectant spray system, ozone spray, formaldehyde vapors or a disinfectant spray and UV light combination. Safety should always be foremost in the minds of those handling unfixed, fresh tissue samples.

Rapid biopsy for intraoperative diagnosis

Frozen sections provide a valuable tool in the rapid diagnosis of tissues during surgery. The pathologist selects a piece of tissue and this is frozen using one of the techniques previously discussed. The correct orientation of the tissue cannot be over emphasized. The slide is immediately submerged in cold acetone or 95% alcohol and the sections are stained immediately by a rapid hematoxylin and eosin (H&E), methylene blue or polychrome stain. With properly cut and stained slides a rapid diagnosis can be made for the surgeon. Tissue samples or the area of interest may be small and care should be taken to provide a slide of diagnostic quality.

Ultracryotomy

This is used primarily in research laboratories. It involves rapid freezing of fixed or unfixed tissue by using isopentane and liquid nitrogen and cutting

sections at 50-150 nm. Success has been gained by pre-fixing the tissue in glutaraldehyde prior to sectioning.

Equipment

There are two basic types of equipment for frozen ultra-thin sectioning. The first uses a standard or slightly modified ultra-microtome in a deep freeze, and the second utilizes a microtome specially designed for ultra-thin sectioning at low temperatures. The temperature control on these ultra-cryotomes is between −20 and −212°C. The sections are cut with a glass knife and picked up on grids. A cutting temperature of approximately −180°C is suitable for most tissues. These sections are useful in the localization of enzyme activity at the ultrastructural level.

Freeze drying and freeze substitution

Freeze drying is the technique of rapid-freezing (quenching) of fresh tissue at −160°C, and the subsequent removal of water molecules, in the form of ice, by sublimation in a vacuum at the higher temperature of −40°C. The blocks are then raised to room temperature and fixed by vapor or embedded in a suitable medium. This technique is usually restricted to research laboratories. The technique minimizes:

- Loss of soluble substances.
- Displacement of cell constituents.
- Chemical alteration of reactive groups.
- Denaturation of proteins.
- Destruction or inactivation of enzymes.

Applications and uses of freeze-dried material

Initially, the technique of freeze drying was used as a method of demonstrating fine structural details. Other applications now include:

- Immunohistochemistry methods.
- Demonstration of hydrolytic enzymes.
- Fluorescent antibody studies: many polypeptides and polypeptide hormones are better demonstrated on freeze-dried sections.
- Autoradiography: excellent results are obtained with freeze-dried sections with accurate localization of soluble substances. Water-soluble isotopes can be used if the sections are dry-mounted onto the slide.
- Microspectrofluorimetry of autofluorescent substances requires sections which are unaltered by processing methods. Frozen sections give adequate results but are affected by thawing of the water in the section. The effect of the embedding medium on freeze-dried sections appears to cause less damage.
- Formaldehyde induced fluorescence (FIF) is used in the demonstration of biogenic amines. This involves the use of blocks of tissue which are freeze-dried and subjected to formal vapor at a temperature of 60 to 80°C under controlled humidity. FIF is used to demonstrate 5-hydroxytryptamine, epinephrine (adrenaline), norepinephrine (noradrenalin) and other catecholamines. When these amines react with formalin they are converted to fluorescent compounds.
- Mucosubstances show good staining after freeze drying followed by formal vapor fixation. The reactivity of mucins appears unaltered and the localization is improved compared to frozen or paraffin sections. The use of freeze-dried sections is recommended for the accurate demonstration and localization of glycogen.
- Proteins may be satisfactorily demonstrated on suitable fixed freeze-dried sections. Formal vapor fixation may remove some protein in sections.
- Scanning electron microscopy shows reduced distortion of the tissue with standard processing if the tissue is then freeze dried.

Frozen section substitution

The technique of frozen section substitution involves the rapid freezing of the tissue to −160°C in isopentane super cooled by liquid nitrogen. Cryostat sections are cut at 8–10 µm and placed in a cold container maintained at cryostat temperature. The sections are transferred to water-free acetone and cooled to −70°C for 12 hours. The sections are floated onto slides, allowed to dry and the required histochemical method applied. For most diagnostic purposes these cryostat sections preserve most tissue components. This method is convenient, labor-saving and is easy to implement in the laboratory.

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8 Resin (plastic) embedding for microscopy and tissue analysis

John W. Stirling • Anthony E. Woods

Introduction

Resins are used to provide exceptionally strong support in tissue preparation for microscopy. Currently available resins include epoxy and acrylic formulations which can be used in standard histological techniques, but also may be useful in specialized techniques such as immunohistochemistry. The applications, advantages and disadvantages of these agents are described and a comprehensive listing of commercially available resins and resin kits is also included.

The use of resins

Since the introduction of resins into microscopy in the 1950s and 60s (Glauert, 1975), their use has expanded, primarily for electron microscopy. Current techniques and procedures which utilize resins are similar to the basic methods developed in this early period although some methods, and their associated technologies, have been significantly improved, e.g. cryotechniques and microwave-assisted rapid processing and embedding. In addition to their use in electron microscopy, resins are now used in a broad range of techniques and strategies for investigating tissue morphology and tissue components at the molecular level. These include light microscopy, correlative light and electron microscopy, tomography, super-resolution fluorescence microscopy, cytochemistry (histochemistry) and affinity labelling.

Principally, resins are used when there is a need for strong, robust sections which preserve and support the two or three dimensional structure of the tissue and/or the integrity of its molecular constituents. The specific properties of the various resins which make them ideal for this purpose vary depending on the application concerned.

Overall, their general characteristics include many desirable and necessary features:

- Solubility in a range of solvents, e.g. water, alcohol or acetone.
- A range of viscosities are available.
- They polymerize uniformly and in a variety of ways, including at low temperature, but are not usually subject to significant dimensional changes during polymerization.
- Variable hardness but with good plasticity and flexibility so section reasonably easily.
- They lack internal structure and are transparent to light and electrons.
- Some may be etched or removed from tissue sections similar to wax.
- Many are stable under electron bombardment.

In respect to diagnostics and for routine histopathology, commercial paraffin wax and paraffin wax-polymer mixtures are still the most convenient embedding media for the majority of tissues. However, for the methods above, and for some diagnostic applications, resin embedding is superior in some cases and essential in others. Firstly, wax may not give enough support when the tissue and the embedding medium differ significantly in hardness. For example, with un-decalcified bone, especially when the sample is large or dense cortical bone is present, sectioning can be extremely difficult, often producing only fragmented sections. In such cases a more supportive (i.e. hard) resin embedding medium is required which adheres strongly to the specimen, and sectioning may require a range of specialized techniques and equipment, e.g. a motorized microtome.

Extremely hard material such as stents (vascular implants) or teeth may only be prepared as slices which are ground down (milled) and polished to the required thickness (a ground section). Again, this requires specialized equipment and procedures such as petrographic techniques which are different from those used for conventional microtomy (Williamson, 2015).

Secondly, wax is too friable for the production of very thin sections for high resolution light microscopy; a problem also related to tissue support, and the hardness, plasticity and flexibility of the embedding medium. For example, whilst high-quality paraffin wax-synthetic resin blends allow sectioning at approximately 2 µm, 1–2 µm resin sections of nerves, renal biopsies and hematopoietic tissues are required to detect minor tissue changes which may be obscured in wax sections.

Thirdly, labile substances such as enzymes may be lost during processing and wax embedding. In these circumstances, resin, rather than paraffin wax may be superior, although some resins, which cannot be removed from the section, may create difficulties with respect to other techniques due to resin masking, e.g. in affinity labelling.

Fourthly, wax is completely unsuitable for electron microscopy as it cannot withstand electron bombardment. In contrast, many resin media (epoxies and some acrylics) have high structural integrity and are relatively stable under electron bombardment, minimizing section damage (volume loss and dimensional changes). For electron microscopy and electron tomography this is an essential property: it is critical that the three-dimensional structure of the cell and the integrity of its molecular components are maintained and only cryosections or extremely thin resin sections (approximately 60-500 nm depending on the application) are suitable.

Note that this chapter focuses principally on the use of resins for light microscopy; the use of resins in electron microscopy is described in Chapter 21. For the effects of resins on staining, refer to Chapter 9 and techniques for bone are discussed more fully in Chapter 17. For detailed embedding protocols and additional information on the properties of all resin types see Newman & Hobot (2001), Hayat (2000) and Glauert (1975).

Types of resin embedding media and commercial resin 'kits'

Three types of resin embedding medium are used in histology and electron microscopy and these are classified according to their chemical composition as acrylic (Table 8.1), epoxy (Table 8.2), or polyester resins. These media generally comprise of one or more resin monomers plus additional components such as a hardener, flexibilizer, plasticizer and accelerator (initiator). Some mixtures may also contain a filler and/or a regulator e.g. Technovit T9100. The various components may be supplied individually or together as a kit. A small number of resins are supplied as 'pre-mixed' single solution kits. The polyester resins (Vestopal) are no longer in common use.

Prior to use, the resin monomers and other components are mixed together in specific proportions, the ratios being changed to alter the physical and chemical properties of the liquid resin mixture, the mode of polymerization (i.e. the change from liquid resin mixture to solid block), and the physical and chemical properties of the final polymerized block. Importantly, when using multicomponent resin systems, it is critical to ensure that all the components are accurately measured and thoroughly mixed in order to produce good quality blocks with a consistent internal composition. It should be noted however, that the infiltration rate of each resin component will depend on tissue density and the size of the diffusing molecules and that, after mixing, when the polymerization process has been activated, the resin may start to increase in overall viscosity. Longer infiltration times are required for more viscous resins. It follows that, if tissue infiltration is incomplete for any reason, polymerization may not be uniform.

Depending on the resin involved, and the imaging and labelling techniques to be applied, polymerization may be carried out chemically, or by using heat (a standard or microwave oven) or light (UV or blue light). Resins which cure with an exothermic reaction may need to be cooled or polymerized in the cold (approximately 4°C or less). Oxygen-sensitive resins, mainly acrylics, must be polymerized in a vacuum, in an inert gas atmosphere e.g. dry

Table 8.1 Acrylic resins

The following are the principal characteristics and applications of the main commercial acrylic resins and resin kits available from a selection of major histology and electron microscopy laboratory suppliers. Note that there may also be additional proprietary resins available with similar components and specifications. For full technical, processing and application details (including references to key articles), refer to the technical data sheet for the specific resin, or the supplier/manufacturer's website.

Resin	Main techniques	Main characteristics
Technovit®7100 *Glycol methacrylate*	LM 1 µm serial sections Enzyme histochemistry	Colorless block Polymerization method: C (~40°C) ± Oxygen sensitive (unsealed embedding)
Technovit® 8100 *Glycol methacrylate*	LM 1 µm sections Enzyme histochemistry Immunohistochemistry	Colorless block Polymerization method: C (~4° and 40°C)
Technovit® 9100 *Methyl methacrylate*	LM Enzyme histochemistry Immunohistochemistry In situ hybridization Mineralized tissues (heavy duty microtome) Cutting and grinding techniques	Compatible with most routine LM stains Polymerization method: C (-8 to -20°C) Oxygen sensitive
LR White	LM and EM Immunohistochemistry Decalcified bone and teeth Materials science	Low viscosity (8 cps) Hydrophilic Medium and hard grade Polymerization temperature: 0 to 5°C (exothermic) Polymerization method: C, MW, UV Oxygen sensitive
LR Gold	LM 1–4 µm sections and EM Enzyme histochemistry Immunohistochemistry Compatible with unfixed tissue and low temperature techniques (free-floating sections); may not be suitable for dark tissues	Use polyvinyl pyrrolidone (PVP) to protect unfixed tissue from osmotic damage May be cured at temperatures down to -25°C using a visible light source and benzil Polymerization method: C, L (blue light), UV Oxygen sensitive
JB-4™ *Glycol methacrylate*	LM 0.5–2 µm sections High resolution LM Lipid and enzyme retention (cold technique) Calcified bone Embryonic tissues	Water soluble, complete dehydration through 100% alcohol not required Less shrinkage than wax sections Rapid cure : 90 mins Polymerization method: C (0°C to room temperature, exothermic)
JB-4 Plus™ *Glycol methacrylate*	LM Similar to JB-4™ Dense specimens (bone) Temperature-sensitive specimens	Harder blocks than JB-4™ Low temperature polymerization; less exothermic than JB-4™ Polymerization method: C
Osteobed *Methyl methacrylate*	LM Large and small mineralized (undecalcified) bone specimens Immunohistochemistry	Large samples: cut with a heavy duty microtome Resin can be removed from the section: staining procedures similar to paraffin sections Polymerization method: C (32–34°C, exothermic)

Table 8.1 Acrylic resins—cont'd

Resin	Main techniques	Main characteristics
Acrolysin (hard) *Methyl methacrylate*	LM 200 µm sections Thick section histology: diamond saws, wires, circular disks and grinders are used for sectioning	Soft and hard formulations Resin can be removed from the section similar to paraffin sections Polymerization method: C (room temperature: 21–22°C, exothermic)
Acrolysin (soft) *Methyl methacrylate*	LM 4–10 µm sections Undemineralized bone, hard tissues, and porous bio-materials/implants. Techniques where motorized rotary and sledge/polycut microtomes are used for sectioning Enzyme histochemistry Immunohistochemistry	Resin can be removed from the section similar to paraffin sections Polymerization method: C (room temperature: 21–22°C, exothermic)
Histocryl	LM 1–5 µm sections Routine LM Hard tissues	Can be sectioned on a steel knife; motorized microtome/glass knife best 'Water clear', hydrophilic; most routine LM stains (no etching) Polymerization method: C (exothermic)
Lowicryl K4M	LM and EM Temperature-sensitive tissues and techniques, freeze substitution	Hydrophilic Use down to -35°C Polymerization method: C; UV Oxygen sensitive
Lowicryl K11M	LM and EM Temperature-sensitive tissues and techniques, freeze substitution	Hydrophilic Use down to -60°C Polymerization method: UV Oxygen sensitive
Lowicryl HM20	LM and EM Temperature-sensitive tissues and techniques, freeze substitution	Hydrophobic Use down to -70°C Polymerization method: C; UV Oxygen sensitive
Lowicryl HM23	LM and EM Temperature-sensitive tissues and techniques, freeze substitution	Hydrophobic Use down to -80°C Polymerization method: UV Oxygen sensitive
Lowicryl Monostep K4M & HM20	LM and EM Immunohistochemistry	Pre-mixed single component kits with the same specifications as the full kits
Methyl/butyl methacrylate mixture	LM 1–3 µm sections and EM Normal and mineralized specimens	Adjustable, soft to hard Resin shrinks during polymerization Can be removed with acetone for LM staining Polymerization method: C; UV
Unicryl™ (Bioacryl)	LM 1–3 µm sections and EM Cytochemistry Immunohistochemistry In situ hybridization	Adjustable, soft to hard Sectioning cleaves block to expose tissue components at surface Resin shrinks ~10% during polymerization Can be removed with acetone for LM staining Exothermic Polymerization method: C (50–60°C); UV (-10–20°C)

Continued

Table 8.1 Acrylic resins—cont'd

Resin	Main techniques	Main characteristics
Micro-Bed *Acrylic-polyester mixture*	LM and EM Immunohistochemistry	Single component resin, water soluble Hydrophilic Non-cross-linking Similar to wax with most routine LM stains including H&E; polychrome silver-based and cytochemical stains (PAS) also feasible Polymerization method: C (50–60°C); UV (-10–20°C)
Immuno-Bed ™ *Glycol methacrylate based*	LM Immunohistochemistry	Low viscosity Semi-soluble, may be partially removed Good with routine LM stains; antibodies can penetrate resin Infiltration, embedding and sectioning procedures are similar to JB-4™ Polymerization method: C (4°C or in a cold room, exothermic) Oxygen sensitive
Glycol methacrylate and low-acid glycol methacrylate	LM 1–3 µm sections and EM Cytochemistry Immunohistochemistry Autoradiography	Sections of ~1 µm can be cut with a rotary microtome and steel knife (or glass) and stained with a variety of special stains Thin sections may expand rapidly on water Low-acid version may give improved immunolabelling Water miscible, hydrophilic Polymerization method: UV (4°C or in a cold room, exothermic) Oxygen sensitive (embed in closed gelatin capsules)
Glycol methacrylate and polyethylene glycol (PEG)	LM 1–2 µm sections and EM Cytochemistry Enzyme histochemistry Correlative LM/EM studies (thick and thin sections of the same block)	Water miscible Alternative formulations with/without the polyethylene glycol Polymerization method: UV Oxygen sensitive (embed in gelatin capsules)
2-hydroxypropylmethacrylate (HPMA)	LM and EM Cytochemistry Soft and hard tissues	Water soluble: resin acts as dehydrant Water reduces brittleness of blocks May be used with divinyl benzene as a cross-linking agent or PEG to give a lower viscosity mixture (faster penetration, easier polymerization) Polymerization method: UV (3°C); C (60°C) Oxygen sensitive (embed in gelatin capsules)

cps: centipoise; EM: electron microscopy; LM: light microscopy
Polymerization method: chemical accelerator (may also require heat) (C); light (L); ultraviolet light (UV); microwave oven (MW)
Compiled from: www.ScienceServices.eu (accessed December 2017); www.emsdiasum.com (accessed December 2017); www.polysciences.com (accessed December 2017); www.dornandhart.com (accessed December 2017); www.bbisolutions.com (accessed December 2017); www.agarscientific.com (accessed December 2017).

Table 8.2 Epoxy resins

The following are the principal characteristics and applications of the main commercial epoxy resins and resin kits available from a selection of major histology and electron microscopy laboratory suppliers. Note that there may also be additional proprietary resins available with similar components and specifications. For full technical, processing and application details (including references to key articles), refer to the technical data sheet for the specific resin, or the supplier/manufacturer's website.

Resin	Main techniques	Main characteristics
Epo-Fix	LM Materials science and metallographics Hard samples & complicated shapes Vacuum embedding	Low viscosity Good specimen adherence Polymerization method: C (RT 8 hrs; 60°C 2 hrs)
Epon 812 *(Embed 812, Ladd LX112)* Embed 812/DER 736: *low viscosity version of Embed 812*	EM (LM)	Polymerization method: C
Hard-Plus resin 812 *Modification of Epon 812*	EM (LM)	Lower viscosity than Epon 812 Intermediate solvent not required for embedding Polymerization method: C
Araldite CY212 (*Araldite M*) *Diglycidyl ether of bisphenol A*	EM (LM)	Viscosity: 1300-1650 cps (25°C) Polymerization method: C
Araldite 502	EM (LM)	Viscosity: 3000 cps (25°C) Polymerization method: C
Araldite 6005 (*GY 6005*) *American Araldite*	EM (LM) Hard specimens which do not require the resin to infiltrate the specimen (slow penetration)	Penetrates slowly Blocks slightly harder than Araldite 502 Polymerization method: C
Araldite/Embed 812 (*Epon 812*)	EM (LM)	Hard blocks/high image contrast Blocks easily sectioned Polymerization method: C
DER 332/732™ *Liquid reaction product of epichlorohydrin and bisphenol A*	EM (LM)	3 mixtures: soft block/hard block (collagenous tissue)/very hard Polymerization method: C
Quetol 651 *Ethylene glycol diglycidyl ether*	EM (LM) Immunohistochemistry	Water miscible, acts as a dehydrant Water reduces cross-linking Polymerization method: C
Durcupan ACM *Aromatic polyepoxide* *Variation of Araldite casting resin M*	EM (LM) Alternative to methacrylate	Low viscosity, low shrinkage Polymerization method: C
Durcupan *Aliphatic polyepoxide*	EM (LM)	Water soluble Polymerization method: C
UltraBed *Modified Spurr's formulation*	EM (LM)	Low viscosity: 65 cps (25°C) Polymerization method: C
ERL 4221 *(Spurr's)* *Replacement for VCD (ERL 4206)*	EM (LM)	Low viscosity Hardness adjusted using flexibilizer DER 736 Polymerization method: C

cps: centipoise; EM: electron microscopy (brackets indicate the resin is not specified for this use, but may be suitable); LM: light microscopy; RT: room temperature

Polymerization method: chemical accelerator (may also require heat) (C)

Compiled from: www.ScienceServices.eu (accessed December 2017); www.emsdiasum.com (accessed December 2017); http://www.taab.co.uk/pdf-products.php?cat_id=2&sub_cat_id=19&pdf_id=6 (accessed December 2017); http://www.polysciences.com/default/catalog-products/life-sciences/ (accessed December 2017).

nitrogen or in sealed embedding molds which are impermeable to atmospheric oxygen. In addition, low viscosity oxygen-sensitive resins should not be mixed vigorously, or for long periods, to avoid drawing oxygen into the resin.

A wide range of resins are now available from the major histology and electron microscopy laboratory suppliers. The diversity of commercial products reflects improvements in resin technology and an increase in the range of applications which utilize resins. While this means that there are now resins designed for particular techniques, the diversity of products (especially acrylics) can make it difficult to identify the best resin and/or formulation for any specific application. The problem is exacerbated by the fact that several proprietary kits may claim to be suitable for the same application because some formulations are marketed under different names (especially the Technovit range) (Hand, 1995a). Furthermore, in some cases the original resin monomers described in the literature may no longer be available and commercial kits contain substitutes (e.g. Epon 812 and Spurr resin ERL 4201 – see Glauert, 1975).

Resin formulations may be designed principally for either light or electron microscopy, or both. The latter (e.g. LR White and Lowicryl K4M) facilitates combined light and electron microscopy examination of the same block or, in correlative microscopy, the same section (Bowdler et al., 1989). Epoxy resins are favored for routine electron microscopy as they cross-link with the specimen, provide excellent ultrastructural preservation and are stable in the electron beam.

Acrylics are preferred for light microscopy, although many can also be used for electron microscopy and for cytochemical and affinity labelling techniques. The acrylic resins best suited to cytochemical and affinity labelling are likely to be those which can be polymerized at low temperature (or with a low polymerization exotherm), thus minimizing protein degradation and damage to cellular structures. In this respect, some acrylics are compatible with vitrified specimens and freeze substitution techniques, properties also desirable for correlative studies and super-resolution fluorescence microscopy (Brown et al., 2010). Resins which do not cross-link with the specimen or require alcohol dehydration are also useful for cytochemical and affinity labelling techniques, as these too can damage cellular components and hinder tissue-probe interactions. Some acrylic and epoxy resins can be easily etched or removed from the section, thus reducing tissue masking and improving access to cellular components.

When resin media were first introduced, knowledge of the chemistry and interactions of the various components was critical for optimizing results. Commercial resins now come with recommendations on their formulation and use for specific applications, as well as details of their chemical and physical characteristics. Some formulations can also be easily modified to change the hardness of the final block. Note that many resin components are toxic and/or hazardous and present potential health and safety problems (particularly contact dermatitis). It is important that all the chemicals used in resin media are handled in accordance with local workplace safety regulations.

Cryotechniques

Cryotechniques are used with fixed and unfixed tissues to immobilize tissue components and improve cell preservation especially for tomography, super-resolution fluorescence microscopy, cytochemistry and affinity labelling studies. Epoxy and acrylic resins can both be used as freeze substitution media. Schedules for epoxy resins and acrylics (LR White, Unicryl and the Lowicryl series) are given by Newman & Hobot (2001); methods for epoxy resin (Epon 812/Araldite 506) and the Lowicryl series are given by Hayat (2000). A method for correlative super resolution fluorescence and electron microscopy using cryo-fixed samples embedded in Lowicryl HM20 is described by Johnson et al. (2015).

Super resolution fluorescence microscopy, correlative microscopy and tomography

Super resolution microscopy enables the visualization of single molecules through fluorescent imaging. For reviews, see: Brown et al. (2010); Nienhaus & Nienhaus (2016). For this application, Brown et al. (2010) used high pressure frozen tissue

which was freeze-substituted and embedded in LR White at -20°C. As the shelf-life of the resin with added catalyst for use at this temperature is reduced to about one month, the resin is normally stored without the catalyst which is added just prior to use (Brown et al., 2010; Newman & Hobot 2001). Sections are cut at 100 nm. For a correlative study, Johnson et al. (2015) used Lowicryl HM20 monostep resin to produce sections which were each examined by both super resolution and electron microscopy.

Transmission electron tomography (TEM tomography) is used to investigate the 3D architecture of the cell in sections 200–500 nm thick. A focused ion beam scanning electron microscope (FIB-SEM) is used to image larger volumes (Kizilyaprak et al., 2015). The stability of the sample and embedding medium, i.e. resistance to mass loss and 3D shrinkage, is extremely important in this technique and the choice of resin is critical. In a comparative study of standard and modified formulations of epoxy resins (Epon, Durcupan, Epon/Durcupan mixtures and Hard Plus 812) and methacrylates (Lowicryl HM20, K11M, HM20/K11M mixtures), Kizilyaprak et al. (2015) found that Hard Plus with 10% linear shrinkage and 15% mass loss was the resin of choice. For immunolabelling studies and correlative fluorescence light microscopy, Lowicryl K11M or K11M/HM20 were a good alternative.

Rapid embedding

Rapid resin embedding and polymerization are extremely useful for the speedy turnaround of diagnostic material and to reduce the deleterious effects of liquid resin, e.g. lipid extraction on tissue samples (Newman & Hobot, 2001). General schedules for epoxy and acrylic resins are described by Newman & Hobot (2001) and Hayat (2000). Webster (2007) describes microwave-assisted techniques. The embedment of frozen tissue into epoxy and acrylic resins using centrifugation for rapid resin infiltration, followed by high temperature polymerization, is described by McDonald (2014). One should note that this novel technique may possibly be adapted for routine samples.

Sectioning resin-embedded material

The majority of resin media require the use of a motorized microtome and specially designed blades rather than standard disposable steel blades in order to cut good quality sections (see also Chapters 7 and 17). The complete range of knives, varying by composition, coating, size and profile available for resin histology and their specific applications is too extensive to be fully discussed here. Full details can be found on the various manufacturer and supplier websites. Briefly, tungsten carbide knives are suitable for acrylic resin applications, including bone, hard materials, stents and for frozen sectioning. Some metal knives are coated with a material such as amorphous diamond, teflon, polymer, ceramic or titanium nitride to reduce friction and increase the longevity of the cutting edge. Diamond-coated metal knives are designed specifically to cut hard materials and various sizes are now made for both light and electron microscopy. Sapphire knives are a cheaper alternative. However, the knife edge of both diamond and sapphire is fragile and easily damaged and, while both will cut resin blocks, neither are suitable for hard material or tissue with hard inclusions. Most resins can be cut on an ultramicrotome with a triangular Hartmann-Latta glass knife (as used in electron microscopy) providing the block is not too large. Rectangular Ralph glass knives, with a long cutting edge are used for standard microtomy. All glass knives are quickly damaged by hard material or inclusions.

Acrylic resins

The acrylic resins used for microscopy are esters of acrylic acid (CH_2··CH·COOH) or, more commonly, methacrylic acid (CH··C(CH_3) ·COOH) and are commonly referred to as acrylates and methacrylates respectively. Some acrylics are water miscible and can be used without the use of a dehydrating solvent. Numerous formulations have been devised with a wide range of properties and applications (Table 8.1). Conventional acrylics are polymerized (cured) by complex free-radical chain reactions, the

radicals usually being produced from the breakdown of a catalyst, most commonly benzoyl peroxide. Radicals can also be produced spontaneously by light or heat so acrylic resins should be stored in dark bottles in a cool place.

Benzoyl peroxide breaks down at 50–60°C but the addition of a tertiary aromatic amine (such as *N*,*N*-dimethylaniline or dimethyl *p*-toluidine) causes it to produce radicals at 0°C and this allows polymerization at low, or room temperature. Dry benzoyl peroxide is explosive and is supplied damped with water as a paste mixed with dibutyl phthalate, or as plasticized particles. For use in some formulations the water must be removed and care must be taken to dry aliquots away from direct sunlight or heat. Light-sensitive photocatalysts such as benzil and benzoin are used for polymerization of acrylics at sub-zero temperatures using short wavelength light.

In addition to the monomer and catalyst, several other ingredients are often necessary in acrylic resin formulations. Amines cause polymerization to proceed at a faster rate and are termed as either accelerators or, more rarely, as activators. Other accelerators include sulfinic acid and some barbiturates. To improve the sectioning qualities of acrylic blocks, softeners or plasticizers are often added to the mix. Typical examples are 2-butoxyethanol, 2-isopropoxyethanol, polyethylene glycol 200/400 and dibutyl phthalate. Some acrylic mixes need a small amount of a cross-linker such as ethylene glycol dimethacrylate to stabilize the resin and protect it from the physical damage which may otherwise be caused by an electron beam, e.g. Lowicryl resins, or staining solutions, e.g. Technovit 8100. Unlike epoxy resins, the viscosity of acrylics is low and whilst relatively short infiltration times are possible, the size and nature of the tissue, together with the processing and embedding temperature, will affect the length of incubation required.

2-hydroxyethyl methacrylate (HEMA), more commonly known as glycol methacrylate (GMA), is a popular embedding medium for light microscopy since it is extremely hydrophilic and allows the use of a wide range of tinctorial staining methods. GMA also sections well providing the block is kept dry. Various mixes have been described and commercial kits are available. Most mixtures are based on the formulation published by Ruddell (1967). Although the mixes all contain GMA, the proportion and variety of the monomer and other ingredients may vary; this means that, comparatively, commercial kits may have different characteristics. The monomer can also be contaminated with methacrylic acid which may result in background staining. This can be reduced by purchasing low-acid GMA or a high-quality proprietary kit such as JB-4, JB-4 Plus (Polysciences Inc., USA), Technovit 7100, or Technovit 8100 (Kulzer, Germany). Butyl methacrylate is now rarely used as the main monomer in any histological formulation as it is unreliable and produces considerable tissue artifact during polymerization. However, butyl methacrylate can be added to some acrylic formulations, e.g. Unicryl (BBI Solutions, Wales, UK) and can be used to modify the hardness of methyl methacrylate blocks.

Some formulations are based on aromatic polyhydroxy dimethacrylate resins (Histocryl, LR White & LR Gold from London Resin Company, UK: see Table 8.1). Histocryl is intended for light microscopy but LR White and LR Gold can be used both for light and electron microscopy as they are hydrophilic and stable in an electron beam. LR White may be polymerized by the addition of dimethyl *p*-toluidine; LR Gold is cured by the addition of benzil and exposure to light from a quartz halogen lamp for low temperature embedding. Other acrylic resins cured at low temperature include the Lowicryl range and Unicryl. The Lowicryl range may also be cured using the photocatalyst benzoin and ultraviolet light. Lowicryl K4M Plus is a light-curable epoxy-acrylate product combining rapid polymerization of the acrylic component with the high strength of an epoxy.

For many years methyl methacrylate (MMA) has been widely used in microscopy because its hardness makes it an ideal embedding medium for undecalcified bone, other hard tissues and tissues with stents or implants. Proprietary kits for these applications include Technovit 9100 (Kulzer), OsteoBed (Polysciences) and Acrylosin (Dorn & Hart Microedge Inc.). Variations of the MMA

formulation allow tinctorial staining and immunolabelling of semi-thin sections for high-resolution light microscopy. Disadvantages of MMA are that it may polymerize with considerable shrinkage and it is a powerful lipid solvent, even at low temperature (Glauert, 1975).

Applications and characteristics

Acrylic resins can be used for both light and electron microscopy and are therefore useful for correlative light and electron microscopy. Some, such as the Lowicryl range, have been developed mainly for electron microscopy (Acetarin et al., 1986) but LR White and Unicryl (Scala et al., 1992) can be used for either. For various technical reasons, not all dual-purpose resins are practical for routine high resolution light microscopy studies.

Hydrophilic resins such as GMA and LR White allow tissue to be stained without removal of the embedding medium and are popular for routine use. Many 'simple' staining techniques may be applied, but some may require modification of the standard method for paraffin sections. All acrylic hydrophilic media are insoluble, consequently all staining occurs with the resin in situ. This can cause two problems: either the medium itself becomes stained, or the matrix acts as a physical barrier, masking the tissue. As noted earlier, acrylic resins may be polymerized using a chemical accelerator, heat, or light and an advantage of some acrylics is that they can be polymerized at low temperature (down to -80°C). The optimal polymerization method will depend on factors such as the imaging, staining and labelling techniques required, the technical practicality of the method, and the equipment available (particularly in respect to low temperature techniques and oxygen-sensitive formulations). Note that resins with an exothermic polymerization reaction may need to be cooled or polymerized in the cold (approximately 4°C or less).

Tinctorial staining

Acrylic sections stain easily and are therefore popular for high-resolution light microscopy; excellent results can be achieved with GMA formulations as well as resins such as LR White, even though the resin cannot be removed. Numerous, but not all, routine histological staining methods for paraffin sections may be applied to sections, e.g. H&E, PAS, van Gieson, alcian blue, Perls', elastic methods, Giemsa, and silver techniques for reticulin, although some methods may need to be modified. For example, media in the London Resin range (Histocryl, LR White, and LR Gold) are all softened by alcohol; this means that alcoholic staining solutions such as those used in elastic methods can easily result in section loss. Consequently, even hematoxylin staining of a London Resin should be progressive to avoid exposure to acid alcohol during differentiation; regressive staining of GMA sections is possible with care. The embedding medium, especially GMA, may also stain, although in some techniques this can be reduced by careful washing. Penetration of stains into GMA and the level of background staining and its removal, depend on the molecular size of the dye and the level of resin cross-linking. Dyes with large molecules (MW approximately 1000) penetrate and are lost slowly; small dyes (MW < 550) penetrate, and are lost quickly. Increased cross-linking inhibits stain penetration (Gerrits et al., 1990). The hydrophilic/lipophilic character of the staining reagent also affects the level of background; lipophilic stains producing intense background staining (Horobin et al., 1992). A numerical guide which helps to avoid artifacts resulting from hydrophobic and size effects is given by Horobin et al. (1992).

In contrast to GMA, MMA can easily be removed prior to staining using similar procedures and solutions to those used routinely for dewaxing paraffin sections, but with slightly extended incubation times. Tinctorial staining of tissue in MMA is possible without removing the resin if no cross-linker has been added; this can be useful for sections of undecalcified bone prepared as described in Chapter 17 (except for semi-thin sections for high-resolution light microscopy).

Enzyme histochemistry

The ability to process, embed and polymerize some acrylic resins at low temperature allows a variety of enzymes to be demonstrated in tissue sections. The use of GMA for enzyme histochemical studies is popular as this resin is easy to handle and produces

good results. Hand (1988) has shown that enzyme activity may be affected during each step of the processing schedule thus, the effects of each step (and the reagents involved) should be tested on the enzyme of interest in order to maximize staining. Although some enzymes are destroyed by routine fixation and processing, a large number have been successfully demonstrated. A simple protocol is to carry out fixation, processing and polymerization at 4°C and then dry the sections onto a coverglass (or slide) at room temperature overnight prior to performing enzyme histochemical staining. After staining, care must be taken to ensure that enzyme diffusion and loss do not occur during the washing and mounting procedures.

A variety of aldehyde fixatives have been advocated for histochemical studies but 10% formal calcium is recommended (Dawson, 1972). Staining may be enhanced if the tissue is subsequently washed at 4°C in 3% buffered sucrose solution. Polymerization is normally carried out at 4°C using a chemical accelerator, but for methods utilizing sub-zero temperatures either an excess of catalyst, or a photocatalyst, can be employed, depending on the resin formulation.

In some cases it may be necessary to avoid fixation. Thompson and Germain (1983) describe a method for processing fresh (unfixed) tissue stabilized with polyvinyl pyrrolidone (MW 44,000) at -25°C and embedded in LR Gold. Polymerization was achieved using the photocatalyst benzil and blue light from a quartz halogen lamp. This procedure has the potential to demonstrate fixation-sensitive enzymes such as the oxidative enzyme succinate dehydrogenase.

Immunohistochemistry

The labelling of antigenic sites in resin-embedded tissue is challenging and the technique is not generally regarded as suitable for routine diagnostics. The effects of fixation, processing and embedding are particularly damaging to epitope reactivity and some epitopes may be chemically altered by the compounds in the embedding medium (Takamiya et al., 1980). Resins which cannot be removed from the section will cause the physical masking of tissue epitopes (Gerrits, 1988). Physical masking is a significant problem as it prevents the penetration and/or attachment of probes in all affinity-labelling techniques. This generally reduces the sensitivity of the technique, often below the detection threshold. While the effects of tissue masking may be at least partially mitigated using antigen retrieval or unmasking techniques (Stirling, 2000) it may be more effective to use alternative techniques. For example, the use of hydrophobic MMA without the addition of cross-linking agents allows the resin to be dissolved and should facilitate labelling. One should be aware that hydrophobic resins such as Lowicryl HM20 and HM23 contain cross-linking agents and are insoluble.

In cases where fixation is a serious problem it may be necessary to use cryopreservation combined with low temperature resin infiltration. An alternative approach is to use inert dehydration combined with embedding in GMA (Stirling et al., 1990). In this technique the unfixed tissue is infiltrated with ethanediol at room temperature and then embedded directly into low-acid GMA. Immunoreactivity remains high with this method but labile components may possibly migrate or be lost. More holistic strategies based on the publications of Beckstead (1985) and Casey et al. (1988) have used 'mild' conditions by fixing, processing and polymerizing the resin at low temperature to protect sensitive epitopes and also to produce a 'looser' embedding matrix which allows antibodies and labelling reagents to penetrate the embedding medium more easily. A number of acrylic resin formulations are available which facilitate antibody labelling (and affinity labelling in general) with some formulations being specifically designed for this purpose (Table 8.1). A novel immunolabelling protocol using GMA has been described by Howat et al. (2005) for tissue microarrays.

A variety of protocols using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen have been developed and these may be combined with DAB amplification. A typical example is that described by Newman et al. (1983) for LR White-embedded material. In this technique the DAB was intensified using a gold-sulfide-silver method. The use of small molecular weight antibodies and chromogens such as aminoethylcarbazole (AEC) are suggested to improve immunostaining with ImmunoBed (Polysciences).

The use of MMA is described by Hand et al. (1989) in which tissue is fixed in formalin under routine conditions then processed and embedded at room temperature. The resin is polymerized using a chemical accelerator, e.g. *N,N*-dimethylaniline, although other amines have been used successfully. The embedding procedure is similar to that used for GMA blocks using open molds in a glass desiccator. The MMA is soluble and is removed prior to immunolabelling. An example of typical immunoperoxidase results using MMA are shown in Fig. 8.1.

For in-depth details of immunocytochemical techniques and protocols that apply to resin-embedded tissues see Griffiths (1993) and Newman & Hobot (2001). Standard antigen retrieval techniques developed for light microscopy may be effective on resin sections and a protocol employing Triton X-100 pretreatment of LR White thin sections to improve immunofluorescence specificity and intensity is described by Ghrebi et al. (2007). For full details of antigen retrieval techniques see Chapter 19 and Shi et al. (2000). Antigen retrieval as applied to resin sections for electron microscopy is reviewed by Stirling (2000).

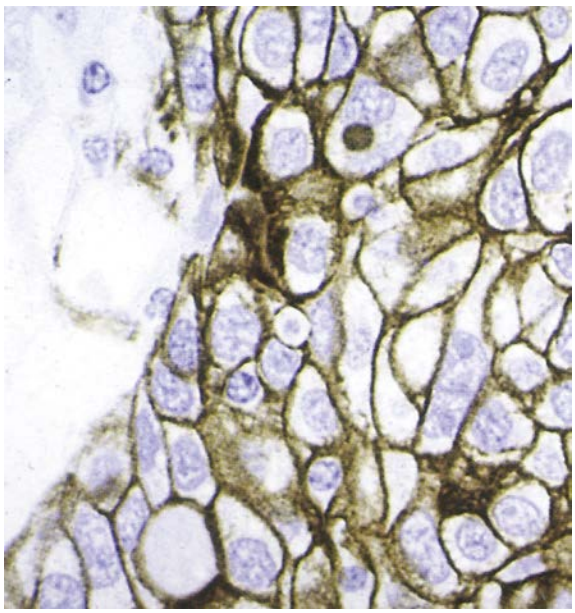

Fig. 8.1 Section of formalin-fixed ovarian tumor embedded in methyl methacrylate showing immunoperoxidase staining of cytokeratin 7 following pretreatment using microwave antigen retrieval. Chromogen DAB. Reproduced, with permission, from Hand, N.M., Blythe, D., Jackson, P., 1996. Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. Journal of Cellular Pathology 1, 31–37. © Greenwich Medical Media Ltd, London.

In situ hybridization

Acrylic resins are compatible with standard in situ hybridization techniques giving high morphological detail combined with the ability to localize low copy transcripts. However, resin embedment does present similar problems to those encountered with immunocytochemical applications, i.e. the effects of tissue processing and physical masking by the embedding medium.

To overcome the problem of physical masking Warren et al. (1998) embedded tissue in a mixture of 80% butyl-methacrylate, 20% methyl-methacrylate, 0.5% (w/v) benzoin ethyl ether, and 10 mM dithiothreitol (DTT). Blocks were polymerized at -8°C. Sections 1.5–2 µm thick were incubated for 10–15 minutes in 100% acetone to remove the resin prior to labelling using a variety of probing and detection strategies. Both mRNA and rRNA transcripts were successfully localized. In some cases, combining resin removal with a brief proteolytic digestion increased the intensity of the hybridization signal with minimal loss of morphology.

Similarly, Torgersen et al. (2009) used Technovit 9100 (an MMA formulation) for both in situ hybridization and immunolabelling studies. Resin removal was achieved using successive incubations in xylene (1 hr), 2-methoxyethylacetate (1 hr), and acetone (10 min). Technovit 8100 has been used successfully without resin removal (Moter et al., 1998). Church et al. (1997) investigated the use of a variety of pretreatments on MMA sections including proteinase-K and heat treatment (pressure cooker and microwave oven). The best results were obtained using the pressure cooker and polymerization of the resin with N,N-3,5-tetramethylaniline. Zhang et al. (2010) describe a low-temperature GMA technique suitable for bone marrow immunohistochemistry, polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) analysis.

Sectioning

For routine purposes 2–3 µm sections of GMA, MMA, and LR White may be cut with a steel knife on a

standard microtome but high-quality sections may require a glass knife (Latta-Hartmann or Ralph knife) or a specialized knife and a motorized microtome. GMA sections will flatten on contact with water at room temperature and can be picked up from a water bath in the same way as paraffin sections. Using the MMA mix described here (see methods), flatten sections on a water bath at 65–70°C. LR White sections are floated out on 70% alcohol or 30–40% acetone on a hot plate at 60°C. Acrylic sections are best picked up on grease-free slides coated with an adhesive such as 2% APES (3-aminopropyltriethoxysilane). Allow the sections to drain for approximately 30 minutes before drying on a hot plate at 60°C. MMA sections may detach from the slide, especially when used for undecalcified bone marrow trephines. Whilst this is true of many MMA formulations, section loss is minimized using the MMA formulation described here (see methods). The use of Superfrost Plus slides without adhesive for bone samples is recommended.

Staining sections

As previously described, GMA and LR White sections can be stained without removing the resin matrix. LR White is softened by alcohol so, after sections are stained, they should be blotted and dried on a hot plate at 60°C for a few minutes before being dipped in xylene and mounted. MMA sections require the resin to be removed prior to staining by immersing the slides in xylene for 10–20 minutes at 37°C. Numerous H&E protocols can be used but the method described below is suitable for GMA and LR White. For MMA sections, 30 minutes in hematoxylin and 5 minutes in 1% buffered eosin staining is preferred and the sections should be washed rapidly in water and ethanol as the eosin is quickly removed.

Acrylic resins: staining sections with H&E

Method

1. Stain in Harris' or Gill's alum hematoxylin for 10–20 minutes.
2. Wash in tap water.
3. If necessary, differentiate in 1% acid alcohol for 2–3 seconds.
4. Blue in tap water.
5. Wash in water for 15 minutes.
6. Stain in filtered 1% aqueous eosin in 1% calcium chloride for 3 minutes.
7. Wash in tap water for 30 seconds.
8. Blot dry.
9. Rinse in absolute ethanol for 20 seconds.
10. Rinse in xylene.
11. Mount in DPX.

Note

Omit steps 3 and 9 for LR White.

Immunohistochemistry using MMA sections

MMA sections can be labelled using routine visualization and amplification protocols (e.g. avidin-biotin and polymer-based techniques) similar to those used for paraffin sections. DAB can also be used as the chromogen and intensification is not required for visualization. Procedures are carried out at room temperature with standard reagents and incubation times. Optimal staining of some antigens may require changes in pretreatment and/or dilution of the antibody. Note that resin sections may dry out faster than paraffin sections.

Similar to wax sections, immunolabelling can be significantly improved using antigen retrieval pretreatment. Enzyme digestion with trypsin has been used for some antigens, but heat-mediated procedures, using either a microwave oven (Hand et al., 1996) or a pressure cooker (Hand & Church, 1998) with sodium citrate solution can significantly improve staining (Fig. 8.1). In general, protocols for resin sections should be similar to those for wax sections although for some antigens the standard technique may need to be modified, or a combination of pre-treatments may be required to maximize labelling. Heat-mediated pretreatment also retrieves antigenicity better in archive material (Hand et al., 1996). For greater detail, refer to Hand, 1995b; Blythe et al., 1997; Hand & Church, 1997. Antigen retrieval techniques are reviewed in full by Shi et al. (2000).

Processing schedules

Only a limited number of schedules are given here as protocols for most media are available as technical data sheets on supplier websites. For examples, see Electron Microscopy Sciences (www.emsdiasum.com) and Ted Pella (www.tedpella.com).

Glycol methacrylate (Ruddell, 1967)

Fixation

Fix tissues in formalin (e.g. formal saline or neutral buffered formalin) or buffered paraformaldehyde.

Solutions

Solution a

2-hydroxyethyl (glycol) methacrylate	80 ml
2-butoxyethanol	16 ml
Dried benzoyl peroxide	0.27 g

Solution b

Polyethylene glycol 400	15 parts
N,N-dimethylaniline	1 part

Processing and embedding

1. If necessary, rinse tissue in an appropriate buffer for 15 minutes.
2. Dehydrate through 70%, 90%, and 100% ethanol. For an average block of 10 × 5 × 2 mm, use two 15 minute changes in each solution.
3. Infiltrate in two changes of solution **a,** each for 1 hour.
4. Embed in a mixture of 42 parts solution **a:**1 part solution **b**.
5. Polymerize at room temperature: stand the molds in cold water to dissipate the heat generated by the exothermic reaction. Polymerization should be complete in 2–4 hours.

Notes

a. Specimens should be processed under a fume hood with extraction.
b. Agitate the specimens continuously during dehydration and infiltration on a roller mixer.
c. Small aliquots of benzoyl peroxide should be dried carefully away from direct heat and sunlight (potentially explosive).The compound must be completely dissolved in the infiltrating solution for approximately 30 minutes.
d. Several block molding systems are commercially available. The open polypropylene molding tray system enables the tissue to be attached directly onto a block stub (Polysciences Inc., USA). To achieve good polymerization, exclude oxygen by using sealed molds in a desiccator filled with oxygen-free nitrogen.
e. Acrylic resins are best prepared in the quantity required, preferably using a large capped glass vial. Measure quantities by volume.
f. Waste solutions containing resin components must be handled and discarded in accordance with local and legal requirements.

JB-4: Modified schedule for hematological bone marrow trephines

Solutions

JB-4 is supplied as a kit containing solution **A** (monomer), solution **B** (accelerator) and plasticized benzoyl peroxide (catalyst).

Fixation

Fix tissues in formalin (e.g. formal saline or neutral buffered formalin) or buffered paraformaldehyde.

Processing and embedding

1. If necessary, rinse tissue in an appropriate buffer for 15 minutes.
2. Dehydrate through 70%, 90%, and 100% ethanol using two 30 minute changes in each solution.
3. Infiltrate tissue in two changes of freshly prepared catalyzed solution **A** (prepared by mixing 120 mg of benzoyl peroxide plasticized in 9 ml of solution **A** and 1 ml of methyl methacrylate monomer: mix until the solid is completely dissolved) for 1 hour, followed by infiltration overnight at room temperature.
4. Embed in pre-chilled embedding medium (10 ml of catalyzed solution **A** and 450 µl of JB-4 solution **B**) at room temperature in a mold. The time needed for full polymerization varies with the temperature, atmospheric oxygen etc. but at room temperature this may be 1–2 hours.

Notes

See the notes in the glycol methacrylate schedule.

Methyl methacrylate

This schedule can be used for routine tinctorial and immunocytochemical staining, although other schedules and mixtures have been published. Care should be taken when handling MMA as it has a pungent odor and is flammable.

Fixation

Fix tissues in formalin (e.g. formal saline or neutral buffered formalin) or buffered paraformaldehyde.

Infiltration solution

Methyl methacrylate monomer (unwashed)	15 ml
Dibutyl phthalate	5 ml
Dried benzoyl peroxide	1 g

Processing and embedding

1. Dehydrate through 50%, 70%, and 90% ethanol. For an average block of 10 × 5 × 2 mm, use 1-hour changes in each solution.

2. Complete dehydration through two changes of 100% ethanol, each for 1 hour.
3. Infiltrate in two changes of infiltration solution, each for 1 hour.
4. Infiltrate in a further change of infiltration solution overnight.
5. Embed in 10 ml aliquots of infiltration solution to which 125 µl of *N,N*-dimethylaniline is added. Polymerization will occur in 3–4 hours. Alternatively, for bone marrow trephines use 250 µl of *N,N*-dimethylaniline to polymerize in 1.5–2 hours.

Notes

a. Aliquots of benzoyl peroxide should be dried carefully away from direct heat and sunlight as it is potentially explosive. No water should be present before dissolving the catalyst in the infiltrating solution for approximately 2 minutes.
b. The notes in the glycol methacrylate schedule also apply.

Epoxy resins

Three types of resin are commonly used in microscopy: those based on either bisphenol A (Araldite), glycerol (Epon and its substitutes) or cyclohexene dioxide (Spurr's) (Table 8.2). Similar to acrylics, epoxy resins are sold as individual components or as proprietary kits which may contain similar formulations. Epoxy resins are widely used, particularly for electron microscopy. In addition to their resistance to electron bombardment, they can be sectioned at approximately 30–40 nm, and the characteristics of the resin and polymerized block can be changed by altering the type and ratio of the resin monomers and the other components. Commercial kits are usually supplied with instructions for making soft, medium and hard blocks. Additional information for changing block characteristics is given by Glauert (1975). Suppliers generally recommend that BDMA (benzyldimethylamine) should be used instead of DMP-30 (2,4,6-tri(dimethylaminomethyl) phenol) as the accelerator for epoxy resins. In comparison to DMP-30, BDMA results in more uniform embedding, it penetrates tissue more easily as it is much less viscous and has a longer shelf-life. Most epoxy resins require an organic solvent such as alcohol or acetone in combination with a transition fluid such as propylene oxide for specimen infiltration, but there are also several water miscible formulations (Table 8.2).

Infiltration by Araldite is relatively slow due to its molecular size. However, the glycerol-based epoxy Epon 812, and its substitutes such as Embed 812 and Poly-bed 812, have a lower viscosity (150-210 cps) and infiltrate faster. The original Spurr's resin ERL 4216 had an extremely low viscosity (7.8 cps at 25°C) but, due to toxicity concerns, this has now been replaced by the more viscous ERL 4221. Current commercial Spurr's resin kits have a viscosity of approximately 65 cps. The current formulations should still penetrate membranes and hard tissues easily and have been used successfully for preparing tissues with a high lipid content. Overall, resin infiltration rates are faster at higher temperatures, although as polymerization proceeds, penetration may slow.

The physical properties of epoxy resins are affected by the way they are polymerized and standard blocks incubated at 60°C are under-cured. Rapid curing for 1 hour at 120°C results in large numbers of cross-links and produces hard brittle blocks. In contrast, curing epoxy resins for 18 hours at 60°C produces blocks which are still quite hard, but easy to section. It should be noted that under-cured blocks may continue to polymerize and that archival blocks may have different properties to fresh blocks which may impact on morphometric studies. Care is also needed to produce blocks with the right level of cross-linking to allow staining. Sodium methoxide can be used to etch resin sections (reducing cross-link density) thus improving access to the tissue for stains and affinity probes.

Epoxy resins have some disadvantages. They are mostly hydrophobic and subsequent oxidation by peroxide to correct this may produce tissue damage. Both epoxide groups and anhydrides can react under mild conditions with proteins and this may reduce the immunoreactivity of tissue epitopes. The components of many epoxy resin formulations

are toxic and commonly cause contact dermatitis. Gloves should always be worn when handling resin components and facilities must be provided for the removal of chemical vapors and the disposal of toxic waste (Causton, 1981). Excess resin can be fully polymerized and subsequently discarded.

Cutting and staining sections for light microscopy

It is not usually possible to cut satisfactory 0.5–1 µm sections with a standard microtome and a steel knife, so sectioning is carried out using a glass, diamond or sapphire knife and a motorized microtome or ultramicrotome. Two types of glass knife are used: a triangular Latta-Hartmann knife or a Ralph knife which has a longer cutting edge (Hayat, 2000). Hard formulations such as Epo-Tech 301, used to embed undecalcified specimens such as teeth, may need to be prepared as ground sections (Williamson, 2015).

Epoxy resin sections are easily stained with aqueous toluidine blue at high alkaline pH. The stain can simply be applied to the section and heated (see Chapter 21). Tissue components stain blue with differing shades and intensities and with no appreciable background staining. Various formulations of polychromatic stains (e.g. Paragon) are also effective and these can be used to simulate H&E staining. Many staining techniques can be applied after the surface resin has been 'etched' using alcoholic sodium hydroxide (Janes, 1979), but the results are not always reliable. Similarly, pre-treatment with potassium permanganate and oxalic acid can be used to oxidize osmium-fixed tissue so that results with aqueous solutions are more consistent (Bourne & St John, 1978).

A few reports have described the application of immunohistochemistry to epoxy sections for light microscopy studies following treatment with sodium ethoxide/methoxide (Giddings et al., 1982; McCluggage et al., 1995; Krenacs et al., 2005). In general, for light microscopy, acrylics are preferred to epoxy resins because of the quality and range of compatible tinctorial stains and they are more amenable to labelling with affinity probes.

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9 Theory of histological staining

Richard W. Horobin

Introduction

The physicochemical mechanisms of most histological stains are now understood. Detailed accounts and general overviews are to be found in the references and further reading at the end of this chapter. Histological staining methods from acid dyes to silver impregnation, involve broadly similar physicochemical principles. The present chapter aims to outline the major theories on common staining procedures and facilitate rational trouble-shooting if problems are encountered.

Key questions to consider when seeking to understand histological stains are:

- Why do *any* tissue components stain?
- Why do stained components *remain* stained?
- Why are *all* components not stained?

These questions can be answered for most stains, although some answers are complex. For instance, enzyme histochemistry, immunostaining and the PAS procedure involve biochemistry, immunochemistry and organic chemistry respectively. However, these methodologies are all influenced by selective uptake of stains and staining reagents into cells and tissues, and selective losses of stains from tissues. Which uptakes and losses occur depends both on binding equilibria and on rate factors.

Nomenclature note: *staining* always involves the visual labeling of some biological entity by attaching, or depositing in its vicinity, a marker of characteristic color or form. The *stain* is the marker, or the reagent used to generate the marker.

Why and how staining happens

Why are stains taken into the tissues?

Stain uptake is often due to dye-tissue or reagent-tissue affinities. In the physicochemical literature, to say a tissue component has a high affinity for a dye merely means there is a tendency for a stain to transfer from solution onto a section and this concept is used here. The magnitude of the affinity depends on every factor favoring or hindering this movement. The familiar stain-tissue attractions, including stain-solvent and stain-stain interactions, can be influential, as can solvent-solvent interactions.

This account initially assumes staining reaches equilibrium, and the consequences of this not being reached are discussed later. Moreover, uptake of dyes and reagents is often multistep in both space and time. Thus, a reagent may initially enter tissues due to coulombic attractions. Once inside it may form covalent bonds with some tissue grouping. The intensity of staining may also be influenced by stain solubility in solvent and tissue environments.

Various contributions to stain-tissue affinity are outlined in Table 9.1 and are discussed below. Practical staining processes commonly involve several such factors. However, as histologists and histochemists often emphasize reagent-tissue attractions as affinity sources, these interactions are discussed first.

Reagent-tissue interactions

Coulombic attractions have been termed salt links or electrostatic bonds, and have been the most widely

Table 9.1 Factors contributing to dye-tissue affinities

Interactions	Practical examples where the factor is important
Reagent-tissue interactions	
Coulombic attractions	Acid and basic dyes, and other ionic reagents, including inorganic salts
van der Waals' forces	Strongest with large molecules such as the elastic fiber stains, and final reaction products such as bisformazans in enzyme histochemistry
Hydrogen bonding	Staining of collagen by Sirius red, glycogen by carminic acid
Covalent bonding	Methods such as the Feulgen nuclear, PAS and mercury orange for thiols
Solvent-solvent interactions	
The hydrophobic effect	Staining systems using aqueous solutions of dyes or other organic reagents, e.g. enzyme substrates
Reagent-reagent interactions	Metachromatic staining with basic dyes, inorganic pigments in Gomori-type enzyme histochemistry, metallic microcrystals after silver impregnation

discussed reagent-tissue interactions. They arise from electrical attractions of unlike ions, e.g. the colored cations of basic dyes and tissue structures rich in polyanions such as phosphated DNA or sulfated mucosubstances (Lyon, 1991; Prentø 2009). However, binding of dye ions to an ionic tissue substrate also depends on charge magnitude, the amount of non-dye electrolyte in the dyebath, electrical repulsions between ions of similar charge, and swelling or shrinking of tissue substrates (Scott, 1973; Bennion & Horobin, 1974; Goldstein, & Horobin, 1974b; Horobin & Goldstein, 1974). These phenomena are relevant for all ionic reagents, not just dyestuffs. For example when using periodate as the oxidant in the PAS procedure, the periodate anions do not readily react with anionic polysaccharides, such as chondroitin sulfate (Scott & Harbinson, 1968). Moreover, even uncharged tissue substrates acquire an ionic character after binding ionic reagents, e.g. during staining of glycogen by the PAS procedure.

Reagent-tissue attractions not depending on isolated electric charges include dipole-dipole, dipole-induced dipole and dispersion forces; collectively described as *van der Waals' forces*. These occur between all reagents and tissue substrates. However, as extensively delocalized electronic systems favor larger dipoles and greater polarizability, van der Waals' forces are more significant when tissues or stains contain such moieties. Consequently, proteins rich in tyrosine and tryptophan residues, and nucleic acids containing heterocyclic bases, favor van der Waals' attractions. This is also true for the large aromatic systems of bisazo dyes and bistetrazolium salts, halogenated dyes such as rose Bengal and phloxine, and indoxyl and naphthyl enzyme substrates (Horobin & Bennion, 1973). For instance, van der Waals' attractions contribute substantially to stain-tissue affinity when staining elastic fibers which are rich in aromatic desmosine and isodesmosine residues with polyaromatic acid and basic dyes such as Congo red and orcein (Horobin & Flemming, 1980).

Hydrogen bonding is occasionally discussed in the biological staining context. This attractive interaction arises when a hydrogen atom lies between two electronegative atoms e.g. oxygen or nitrogen, though being covalently bonded only to one. Water is hydrogen bonded extensively to itself, forming the clusters important for the hydrophobic effect discussed below. This effect also applies to molecules carrying hydrogen bonding groups present in many dyes and tissue components. As water molecules vastly outnumber dye molecules, hydrogen bonding is not usually important for stain-tissue affinity when aqueous solvents are used. Exceptions arise when hydrogen bonding is favored by the substrate, as with collagen tissue fibers (Prentø, 2007). A related attractive phenomenon, halogen bonding (Metrangolo et al., 2005), may also contribute to staining affinity. This could explain strong staining seen with dyes such as eosin Y (4 arylbromo

substituents), phloxine (4 bromo plus 4 chloro), and other halogenated fluoresceins.

Covalent bonding between tissue and stain is yet another source of stain-tissue affinity. This process underpins the commonly used PAS reactive stains, as well as the historic Feulgen nuclear stains. The polar covalent bonds between metal ions in dyes such as hematein and tissue substrates are another possible example. Dye-tissue binding considered to involve such polar bonds is termed *mordanting*. However, this is of uncertain status since the characteristic staining properties of mordant dyes may have other, or additional causes. Unlike most cationic dyes used as biological stains, common cationic metal-complex dyes are strongly hydrophilic (Bettinger & Zimmermann, 1991) and resist extraction into alcoholic dehydration fluids (Marshall & Horobin, 1973).

Solvent-solvent interactions

A major contribution to stain-tissue affinity when using organic reagents or dyes in aqueous solution is the *hydrophobic effect*. This involves no stain-tissue attractions, but is the tendency of hydrophobic groupings (e.g. leucine and valine side chains of proteins; biphenyl and naphthyl groupings of enzyme substrates and dyes) in an aqueous milieu to come together, even though they were initially dispersed. This interaction occurs because water molecules are linked together by hydrogen bonds into transient clusters whose presence is favored by hydrophobic groups. Any process breaking clusters into individual water molecules occurs spontaneously, as this increases system entropy (cf. the second law of thermodynamics). Consequently, removing cluster-stabilizing hydrophobic groups from contact with water by placing them in contact with each other, is thermodynamically favored. Accounts of the hydrophobic effect are provided by biochemists, amongst others (Tanford, 2004). The effect becomes more important as substrate and reagent become more hydrophobic. Thus, when staining fats with Sudan dyes applied from substantially aqueous solutions, the hydrophobic effect provides major contributions to affinity. Although the phenomenon is sometimes termed hydrophobic bonding, no dye-tissue hydrogen bonds are involved.

Staining using Sudan dyes in non-aqueous solvents does not involve the hydrophobic effect. However, as described in chemical thermodynamics texts (e.g. Adamson, 2012), the tendency of a system to change spontaneously to maximize its disorder, and for *entropy* to increase, provides an explanation. Presence of dye in solvent and lipid constitutes a more disordered system than dye restricted to the solution. So dye disperses, and staining occurs. Such increases in entropy involving substrate and stain occur in all types of histological staining.

Stain-stain interactions

Dye-dye interactions can also contribute to affinity. Even in dilute solutions, dye molecules can attract each other, forming aggregates. In aqueous solutions this may be driven by the hydrophobic effect, but in both aqueous and non-aqueous solutions van der Waals' attractions between planar dye molecules occur. Dye aggregation increases with concentration, e.g. when high dye concentrations build up on tissue sections. With basic (cationic) dyes, such as toluidine blue, this occurs on substrates of high negative charge density e.g. sulfated polysaccharides in mast cell granules giving *metachromatic staining*. This color effect arises because dye aggregates have spectral properties different from the monomeric dye. That dye-dye interactions contribute to affinity in tissue sections was demonstrated quantitatively by Goldstein (1962).

Other cases where stain-stain interactions contribute to affinity include metallic nano and microcrystals generated by gold or silver impregnation (Uchihara, 2007), ionic metal sulfide precipitates formed in Gomori-type enzyme histochemistry, and the purple azure-eosin charge transfer complex produced during Romanowsky-Giemsa staining of cell nuclei (Horobin, 2011).

A minor anomaly

Some stains are not taken up by their tissue targets. In *negative staining* the shapes of structures are disclosed by filling or outlining them with a stain. Examples include demonstrating canaliculi in bone matrix using Schmorl's picro-thionine stain

and visualizing individual microorganisms using nigrosine.

Solubility, an unacknowledged factor

The solubility of stains and staining reagents is a key practical property. Thus, when staining fat with a Sudan dye, the upper limit of staining intensity is set by dye solubility in the lipid, and is further influenced by dye solubility in the staining bath solvent. Solubility is also critical for dye retention after staining, as discussed below. Solubility has complex causes but, in general, the stronger the reagent-reagent interactions, the lower the solubility. Physicochemical texts provide overviews of solubility e.g. Letcher, 2007.

Why is stain retained in tissue?

After removal from the staining bath, stain retention occurs if stains have a high affinity for tissue elements and/or low affinity for processing fluids and mounting media, or if stains dissolve in these latter solvents slowly. This is illustrated by the following examples of common stains.

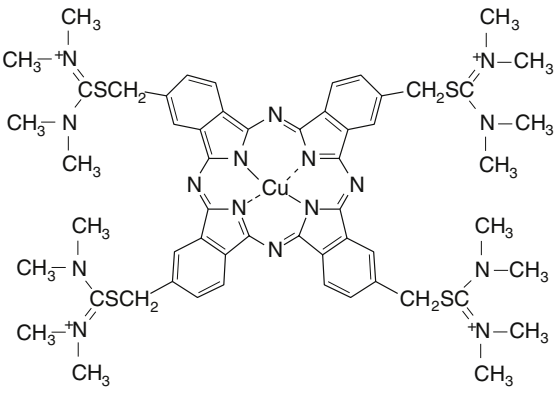
Ionic pigments, such as the Prussian blue generated in the Perls' method for iron, and the lead sulfide produced in Gomori-style enzyme histochemistry are virtually insoluble in solvents used in histotechnology. This is also true for microcrystals of metallic silver and gold produced by metal impregnation. Some organic pigments are less satisfactory. Azo dyes, formazans and substituted indigos produced as final reaction products in enzyme histochemistry have low solubilities in water, but may dissolve in hydrophobic media such as alcohols, xylene or polystyrene. In such cases, hydrophilic mounting media are required, and staining of lipid-rich tissue elements may be artifactual.

Solubilities of azodyes and formazans are sometimes reduced by in situ conversion to metal complexes. Other routine metal complex stains are the aluminum, chromium and iron complexes of hematein, and the chromium complex of gallocyanine. These complexes are poorly soluble in routine processing fluids and mounting media.

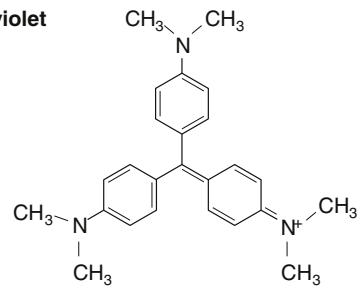
This contrasts with routine basic (cationic) dyes such as crystal violet or methylene blue, which freely and rapidly dissolve in the lower alcohols. Routine acid (anionic) dyes, such as eosin Y or orange G, are often less soluble in lower alcohols, as indeed are hydrophilic basic dyes with large aromatic systems, such as alcian blue. Non-ionic dyes e.g. Sudan stains, are soluble in common dehydrating agents and clearing solvents, as well as in resin mountants. Structures of exemplar hydrophilic and lipophilic basic dyes are shown in Fig. 9.1.

Consequently, sections stained with routine basic dyes must be dehydrated by either passing rapidly through the alcohols, using non-alcoholic solvents or by air-drying. Dehydration is less critical with acid dyes. Sections stained with acid or basic dyes are

Alcian blue 8G



Crystal violet



Dye	Ionic weight	Log P
Alcian blue 8G	1380	–9.7
Crystal violet	372	+1.9

Fig. 9.1 Structural formulae of two widely used basic dyes, plus numerical descriptions of certain of their physicochemical properties. Log P is the logarithm of the octanol-water partition coefficient.

usually mounted in non-aqueous media which do not extract dye. Alternatively, dyes may be immobilized, e.g. by formation of phosphotungstates in the modified Schmorl's method or iodine complexes in the Gram stain. Non-ionic dyes must be mounted in aqueous media.

Why are stains not taken up into every part of the tissue?

The phenomenon of selectivity is crucial for special stains and histochemistry. Even routine oversight stains e.g. hematoxylin and eosin (H&E), Papanicolaou and Romanowsky-Giemsa distinguish nuclei from cytoplasm. The various factors controlling selectivity are discussed below.

Numbers and affinities of binding sites

Both these factors separately influence staining but are not readily distinguished, except by quantitative investigation. Consequently, they are discussed here without distinction.

To understand practical stains it must be appreciated that non-staining of the "background" tissue is as important as staining of the "target" in the material. Sudan dyes provide an example as they have high affinity for fat, but low affinity for the surrounding hydrated proteins. Alternatively, one may consider staining systems in which covalent bonds are formed. Reagents usually give colored products only with a limited range of tissue chemical groupings. Thus, the acid hydrolysis–Schiff reagent sequence of the Feulgen nuclear technique gives magenta derivatives only with DNA. Other examples are provided by traditional anionic dye-cationic dye pairs e.g. H&E, Papanicolaou and Romanowsky stains. The negatively charged acid dyes have high affinities for tissue structures carrying cationic charges i.e. proteins under acidic conditions. However, they have low affinities for structures carrying negative charges e.g. those rich in sulfated glycosaminoglycans or in phosphated nucleic acids. The opposite is the case for basic (cationic) dyes. This produces two-tone staining patterns in which cytoplasm contrasts with nuclear material.

Practical staining protocols maximize selective affinities. Acid (anionic) dyes are applied from acidic solutions, when proteins carry an overall positive charge. Basic (cationic) dyes are applied from neutral or acidic solutions, since under alkaline conditions proteins carry an overall negative charge and also bind basic dyes. Affinities are also influenced by varying inorganic salt concentrations. The selectivity of aluminum-hematoxylins, the critical electrolyte concentration methodology (Scott, 1973) and Highman's and Puchtler's Congo red stains for amyloid all depend on control of electrolyte content.

Rate of reagent uptake

Can structures with equal stain-tissue affinities and equal binding site numbers be distinguished? This is possible if the rate of stain uptake, the rate of subsequent reaction, or the rate of loss of stain are not the same in the different structures.

Progressive staining may be rate controlled e.g. mucin staining using alcian blue or colloidal iron. Selectivity requires short periods of dyeing, during which only fast-staining mucins acquire color (Goldstein, 1962, Goldstein & Horobin, 1974a). After prolonged staining, structures such as nuclei and RNA-rich cytoplasm also stain. Stains used in this way are often large and consequently slow diffusing, so increasing the ease of practical control.

Rate of reaction

Selective staining by reactive reagents may depend on differential *rates of reaction*. For instance, periodic acid can oxidize various substrates present in tissues. However, the histochemical PAS procedure uses short oxidation times, limiting coloration to fast-reacting 1,2-diol groupings of polysaccharides. Enzyme histochemistry provides further examples. When incubating at low pH, hydrolysis of an organic phosphate is rapid in tissues containing acid phosphatases but slow in structures containing alkaline phosphatases.

Rate of reagent loss

Differentiation or *regressive staining* involves selective losses of stain from tissues. Dyeing methods exploiting this include staining muscle striations with Heidenhain's iron-hematoxylin and myelin sheaths with luxol fast blue. In such procedures an initial

non-selective staining is followed by solvent extraction, the dye first leaving permeable structures such as collagen fibers. By contrast, relatively impermeable structures e.g. the A and Z bands of muscle and myelin sheaths, retain stain longest.

Rate control of reagent loss is also important in silver staining of nerve fibers. During impregnation, silver cations bind non-selectively to many tissue sites. Subsequently, the sections are treated with developer which reduces silver cations to silver metal. The rate of this reduction reaction is critical: if too fast because of high concentration or high reactivity of the developer, silver grains are deposited non-selectively throughout the tissue. Whereas if reduction is too slow, no staining occurs because most silver ions diffuse away into the solvent before they are reduced. Selective staining occurs when silver ions diffuse from the background but are retained in less permeable entities e.g. nerve fibers, nucleoli and red blood cells where they are then reduced (Kiernan, 2002; Uchihara, 2007).

Artifacts abound in rate-controlled methods. Any factor influencing rate of reagent entry or loss e.g. section thickness, presence of cavities in the tissue, temperature and stirring of reagent solutions can alter staining results.

Metachromatic staining

Even when neither affinity nor rate controls staining patterns, selective coloration remains possible. For instance, basic dyes such as methylene blue and toluidine blue are absorbed by a variety of basophilic tissue substrates. Chromatin stains "orthochromatically" blue, but cartilage matrix, mast cell granules and mucins stain "metachromatically" reddish purple. This metachromasia is due to dye aggregate formation in the porous, polyanion rich sites (reviewed by Pearse, 1968).

How is staining influenced by tissue fixation?

Fixation is carried out to reduce the non-vital tissue autolysis causing morphological changes, and to prevent losses of some tissue constituents in the processing and staining solutions. This chapter only discusses the influences of fixation on staining.

A given substance may be retained in the specimen to different extents by different fixative agents, and nothing can be stained if it is not retained. For example, many lipids are well preserved after fixation in osmium tetroxide or dichromates, but are poorly preserved after formalin fixation. However, lipids are actively extracted during alcoholic or acetone fixation. Thus, staining lipids after alcoholic fixation is ineffective.

Retention of substances to be stained is necessary, but mere retention may be insufficient. For example, although glutaraldehyde retains more protein than other fixative agents, its use in immunostaining and enzyme histochemistry is limited, even though most antigens and all enzymes are proteins. Chemical reactions insolubilizing proteins also modify haptenic and enzymic activity. Alcohol and acetone, although poor at retaining proteins, are also poor at destroying the activity of whichever antigen or enzyme is retained. Both retention and reactivity of substances affect staining, and both may be fixative dependent.

Fixation also has more subtle influences on staining patterns, acid and basic dyeing provide instructive examples. As shown by Singer (1952), such staining is enhanced by fixative-induced protein denaturation, but the acidophilia-basophilia balance of a tissue is often altered e.g. formalin and osmium tetroxide usually reduce tissue acidophilia, whilst acidic dichromate solutions usually increase tissue acidophilia (Baker, 1958).

What are the effects of specimen geometry on staining?

Here the 'specimen' refers to the biological material in contact with the staining solution, e.g. a dewaxed section or a cervical smear. Such specimens not only have breadth and width, but also thickness and three-dimensional morphology. These features, even at a scale of a few μm or less, influence staining patterns.

Simple geometrical influences

Typically, thin specimens achieve staining equilibrium faster than thick. Specimens with irregular surfaces are stained faster than smooth surfaces, and dispersed cells faster than uniform slabs. Consequently,

in any staining procedure, dispersed specimens such as smears or dabs require shorter staining times than sections of similar cells cut from a solid tissue. Moreover, cryosections usually have irregular surfaces and stain faster than smoother paraffin sections. Resin sections have even smoother profiles than paraffin sections and stain slower, especially when resin remains in situ.

In systems with rate-influenced staining mechanisms, such features modulate selectivity. Some trichrome stains require shorter staining times with cryo-sections than with paraffin sections to avoid overstaining by the higher ionic weight dye.

Effects of more complex specimen geometry

Complex geometries can arise when preparing smears from epithelia. These often contain multicellular clumps of cells, as well as monocellular dispersions. Cells in the middle of such clumps are less accessible to stains than the peripheral cells. Consequently, in rate-influenced methods, such as the Papanicolaou and Romanowsky-Giemsa stains, centrally situated cells can be overstained by the smallest dye present (see Boon and Drijver, 1986, Plates 6.4 and 24.4).

A section's profile may be influenced by fixation. Coagulant fixatives such as Carnoy's fluid tend to shatter cells and tissues, generating more dispersed specimens, whilst fixatives such as formalin give more integral forms (see Horobin, 1982, Fig. 14). Consequently, a rate-influenced trichrome stain which gives the correct color balance when applied to formalin-fixed tissue may overstain with large, collagen binding dyes if applied without modification to material fixed in Carnoy's fluid.

A modification of section geometry induced by microtomy is "chatter". This artifact, related to poor section cutting, produces sections comprised of alternating thick and thin strips. Staining may then generate alternate strips of strong and weak color intensity or, with some trichromes, alternate strips of varying color.

The size of biological structures relative to section thickness can also be significant. Consider secretory granules with diameters much larger or much smaller than the section thickness. All large granules will be sliced through with their contents exposed on a surface of the section, whereas some small granules will be intact and enclosed within the section. This 'access' influences accessibility of larger stains, e.g. macromolecular-labeled antibodies where the 'two types of secretion granule' sometimes reported in immunostaining studies can represent intact versus sliced granules. Such effects are even more pronounced in resin sections.

Geometrical complexity also arises from swelling of cell and tissue components in staining solvents. Materials rich in glycosaminoglycans, e.g. mucus and cartilage matrix, swell markedly in aqueous solutions but collagen fibers swell grossly at extremes of pH. Swelling can increase rates of staining of these structures compared to other material. This contributes to the selectivity of aqueous alcian blue for mucins, with nuclear staining typically being absent after short staining times, and to the selectivity of strongly acidic picro-trichrome stains for collagen fibers. Alcohol does not induce swelling, this may partially account for changes in staining pattern seen when a dye is used from alcoholic rather than aqueous solution. Luxol fast blue, for instance, stains myelin selectively from aqueous solution, but from alcoholic solution gives selective staining of collagen fibers. Such effects are often more marked in resin sections.

What are the effects of resin embedding on staining?

Resin embedding involves infiltration of biological material with a reactive monomer, most commonly an acrylate or epoxide. Subsequent polymerization yields a block of resin enclosing the specimen. Methyl methacrylate resin (MMA) is usually removed prior to staining, after which staining patterns resemble those of paraffin sections. However, glycol methacrylate (GMA) or epoxy resins typically remain in the sections during staining, resulting in different staining patterns to those seen in paraffin wax or cryosections. This is also the case for specimens embedded in a preformed polymer, nitro-cellulose (celloidin). Resulting staining changes have various causes.

Resins can act as stain excluders by obstructing penetration of staining reagents. Resin cross-linking reduces penetration even further. However, resin embedding involves more than mere reduced staining. Resin infiltrates biological specimens unevenly, with dense and hydrophilic structures being poorly infiltrated. For instance, in GMA-embedded specimens, dense or hydrophilic secretion granules are poorly infiltrated, and so stain readily. If the surrounding cytoplasm is well infiltrated with resin, granules may stand out more clearly than in paraffin sections.

Resins can act as stain binders e.g. GMA sections may give background staining with lipophilic dyes such as aldehyde fuchsin. This occurs because the resin itself is slightly lipophilic (Horobin et al., 1992). GMA can also strongly bind to dyes of moderate size, e.g. alum hematoxylins or eosin. These enter the resin and decrease the permeability of the polymer network, so trapping the dye. However, such dye can sometimes be removed by differentiating in plasticizing solvents such as ethanol.

Stain chemistry influences staining patterns: small reagents diffuse rapidly through resins and methods developed for paraffin or cryostat sections can usually be used without modification. When working with routine GMA embedding media, 'small' means <550 Daltons (Da), and includes such common substances as methylene blue, naphthyl phosphate and Schiff reagent. However, large reagents may be totally excluded from resin, restricting staining to resin-free structures. With routine GMA resins, 'large' reagents are those with sizes >1000 Da, e.g. alcian blue, Sirius red and labeled antibodies. The phenomenon of stains binding to lipophilic embedding media noted above occurs only with lipophilic reagents (Horobin et al., 1992).

Some dyestuff properties

General influences of dye chemistry on staining

When physicochemical features of dyes which influence dye-tissue affinity and staining rates are described numerically, systematic correlations can be demonstrated between dye chemistry and staining outcomes. Significant physicochemical parameters include electric charge, the overall size (as represented by ionic or molecular weight) and the hydrophilic/lipophilic character (modeled by the log P value, i.e. the logarithm of the octanol-water partition coefficient). To appreciate the advantages of numerical parameters, see Fig. 9.1, where chemical information concerning two widely used basic dyes is presented in two modes, graphical and numerical.

When considering the relative sizes of dyes, information provided graphically by structural formulae is satisfactory. One can see that alcian blue is a much larger dye than crystal violet. The fact that the staining pattern of alcian blue is highly dependent on staining time (Goldstein & Horobin, 1974a) is therefore not surprising. Unfortunately, the relative hydrophilic/lipophilic character of the two dyes cannot be easily assessed by visual inspection of formulae. However, the log P values of the dyes are clearly different; negative values imply hydrophilicity and positive values lipophilicity. In keeping with this, during alcohol dehydration, sections stained with alcian blue lose no dye, whereas crystal violet is easily lost.

Prediction of stain behavior usually requires consideration of several dye properties. Detailed discussion here is inappropriate, but note that quantitative structure-staining correlations based on such structure parameters can illuminate diverse issues in histotechnology from the staining mechanisms of trichromes (Horobin & Flemming, 1988), to assessment of effects of resin embedding on histochemical staining procedures (Horobin et al., 1992). For overviews see Horobin (2004, 2010).

Effects of dye impurities on staining

Most dye lots used as biological stains are impure. Some contain colored substances not named on the label, or may even lack the named dye. If the colored material is as stated, it may be diluted by colorless materials, e.g. dextrin or an inorganic salt. Moreover, dyes which are pure when purchased may degrade on storage, after being made up into a staining solution, or during staining.

Impurities influence staining in two ways. Firstly, they may alter staining intensity, usually reducing

staining, although occasionally more intense color is seen. Secondly, impurities may change staining patterns by altering the nature and mechanisms of such effects depending on the type of impurity, the staining procedure and the tissue substrate. Unfortunately there is no simple way to identify, and so avoid, such impure products. A practical tip is to purchase dye lots certified by the *Biological Stain Commission*. These have been tested in the Commission's laboratory, and meet purity and staining efficacy criteria. Surprisingly, Commission certified dyes are on average no more expensive than non-certified dyes. For an example of the benefits of certified dyes, see Henwood (2003). Another practical tip is to retain samples of effective dyes. Then, faced with an unexpected staining pattern, to stain the sample with the known effective dye sample, thus ensuring validation. In short, if the known standard dye works, then there may be problems due to dye impurity in the new batch.

If you have an impure dye sample, the most useful advice is to buy another batch of dye, preferably Biological Stain Commission certified. If analysis or purification does prove necessary, an extensive analytical literature may be accessed for individual dyes via the monographs of the 10th edition of *Conn's Biological Stains* (Horobin & Kiernan, 2002), or more generally via an earlier review article by the present author (Horobin, 1969).

Dye nomenclature

Names of individual dyes, and terms used to describe the dye properties, are sometimes inconsistent and/or often confusing. Dyes are complex molecules, nearly all having trivial names which do not explicitly describe their structures. Most dyes used as biological stains were first manufactured as textile dyes when each manufacturer gave a dye their own trade name. A biologist may therefore say 'Use Congo blue', to which his colleagues reply 'But we haven't got any of *that*'- they have, but on their shelves it is labeled trypan blue. Even worse is the surfeit of suffixes. Sometimes these are merely flourishes of a copywriter's pen, so pyronines G and Y are synonyms. Sometimes suffixes indicate dye content, and a standard product may be labeled 'A 100' whilst a grade containing a higher content of dye is termed 'A 150' or merely 'A extra'. Sometimes however, suffixes indicate substantial chemical differences e.g. rhodamines B and 6G describe zwitterionic and cationic dyes respectively.

To reduce confusion, industrial dye users established the *Colour Index* (Society of Dyers and Colourists, 1999). Dyes are given unique code numbers, the *Colour Index* or C.I. number and code names. Thus eosins G, WG and Y are identified as a single dye, C.I. 45380, Acid Red 87; whilst eosin B is a chemically different dye, C.I. 45400, Acid Red 91.

Dyes synthesized for biological staining are named equally idiosyncratically. A traditional example is Gomori's aldehyde fuchsin, and a recent one YOYO-1. Since these products are not of industrial significance, most do not have a *Colour Index* entry. If concerned/confused in such cases one may peruse *Conn's Biological Stains*; see Lillie (1977) and Horobin and Kiernan (2002) for the 9th and 10th editions, respectively. The earlier edition emphasizes traditional stains, the later edition fluorescent probes.

Various terms used to classify dyestuffs are given in Table 9.2. A few comments follow on topics which are sometimes confused in the histochemical literature. *Acid* and *basic* dyes are not acids and bases but salts whose colored species are anionic and cationic respectively. *Neutral* dyes are not non-ionic, but salts in which both the anion and cation are dyes. *Vital stains*, used to stain living cells, are nowadays often called *fluorescent probes* or *biosensors*. One should note that all dyes can be given multiple descriptors. Thus, alcian blue 8G is a *synthetic, basic* dye, structurally a *metal complex*, though not a *mordant dye*, of copper with *phthalocyanine*, substituted by *thioguanidinium groups*, and is routinely used as a *mucin stain*.

Problem avoidance and troubleshooting

Avoiding problems and recognizing errors and achieving a correction are perennial laboratory concerns. The typical strategies are discussed below.

Table 9.2 Some descriptive terms used in classifying dyestuffs used as biological stains

Categories of terms	Examples of terms (and of dyes)
Describing the origins of a dye	Natural (hematoxylin and carmine), synthetic or aniline (almost any other)
Describing physicochemical properties of a dye	Fluorescent (acridine orange), leuco (leuco-methylene blue), metachromatic (toluidine blue), neutral (azure-eosinate)
Giving some kind of description of the dye's structure	Azo (orange G), metal complex (aluminum or iron complexes of hematein), xanthene (pyronine Y)
Describing the dye's usage in biological staining	Fat (oil red O), fluorescent probe (YOYO-1), mucin (alcian blue)
Describing the dye's usage in textile dyeing	Acid (eosin), basic (safranine), direct (Congo red)
Describing the supposed mode of action of the dye	Mordant (gallocyanine chrome alum), reactive (mercury orange)

Strategies for avoiding problems, minimizing the need for troubleshooting

Issues concerning staining procedures

- Use stains compatible with the fixative and embedding medium. *Case example*: water-miscible resin sections do not allow selective staining of elastic fibers with aldehyde fuchsin.
- Use a routine, preferably standardized, staining protocol. *Tip*: see the listing of such protocols at the back of Horobin and Bancroft (1998).
- Use controls proactively to identify problems, not just retrospectively to investigate mistakes. *Tip*: retain samples of stain lots to use if you suspect stain impurity.
- Consider whether you have the necessary skills and knowledge, or if not, who could mentor you. *Tip:* silver stains are tricky; read Kiernan (2002) first, and expect problems with their use.

Issues concerning staining reagents

- Obtain reliable stains and reagents. *Tip*: use Biological Stain Commission certified dyes, which are usually less impure but no more expensive.
- Ensure stains remain reliable. *Tips*: store Schiff reagent in a gas-tight container and dye solutions in lightproof containers.

Cues for recognizing errors - before mistakes can be rectified they must be noticed

- Stain or staining solution is not as expected in terms of color, solubility or stability. *Case example*: some alcian blue samples dissolve, but then precipitate from solution within an hour or less.
- The expected structures stain, but only weakly. *Case example*: unexpectedly weak staining of calcium by alizarin red S can result from aqueous fixatives extracting tissue calcium ions.
- Color of staining is unexpected. *Case example*: excessively red staining seen with Gomori's trichrome may arise from insufficiently acidic staining solutions.
- Unexpected structures stain. *Case example*: granular material stained by the Feulgen nucleal procedure may be carbonate deposits.
- Nature of the staining is unusual. *Case example*: if differential staining of Gram positive and negative organisms is poor, the preparation may be too thick.
- Remember there are always *other* problems! *Case examples*: loss of sections from slides in the Grocott hexamine silver method for fungi due to overheating, and black deposits on slides and sections in the von Kossa procedure due to contaminated glassware.

Once an error has been noticed and a plausible cause identified, corrective action can be taken. A variety of practical problem-solving suggestions for a range of routine and special histopathology stains is provided in Horobin and Bancroft (1998).

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Further reading

Accounts of histotechnology and histological staining rarely describe the physicochemical unity underlying the technical diversity of the various staining technologies. Published protocols are legion, critical reviews and summations rare. Encyclopedic texts such as this one, and earlier works such as those of Lillie (1965), Pearse (1968), and Sheehan and Hrapchak (1987) summarize a remarkable amount of information, and provide extensive bibliographies. Some staining manuals integrate theoretical background with procedural information, e.g. Chayen and Bitensky (1991) and Kiernan (2015). A few authors have provided systematized accounts of staining methods from a modern physicochemical perspective; for instance, Horobin (1982, 1988), Horobin and Bancroft (1998), Lyon (1991), and Prentø (2009). Some classic works can also be recommended. Read Baker (1958) for his early integrative account and his elegant English. Read Lillie (1965) for his hard-won personal experience and historical long view. Then read Mann (1902) to be astonished at why it took us so long to follow up his experimental investigations and mechanistic insights. Several modern texts concerning dyestuffs are available including that of Zollinger (2003) which does, unusually, include a substantial section explicitly discussing biological stains and staining.

10 The hematoxylins and eosin

John D. Bancroft • Christopher Layton

Introduction

Hematoxylin and eosin (H&E) is the most widely used histological stain. It is simple to use, easy to automate and demonstrates different tissue structures clearly. Hematoxylin stains the cell nuclei blue-black, showing clear intranuclear detail, whilst eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange and red. Automated staining machines and commercially prepared hematoxylin and eosin solutions are commonly used in today's laboratories for routine staining. Many laboratories now have to adhere to strict ISO standards to gain accreditation from regulating authorities. These standards cover validation of chemicals and stains used within the laboratory and variability between batches of 'in-house' stains. These regulations and the underpinning paperwork have resulted in many laboratories using commercially produced reagents where validation is performed by the supplier (see Chapter 1). Students of histological techniques should have a basic knowledge of the dyes and their preparation techniques in order to troubleshoot and/or modify procedures for specialized use. Hematoxylin may also be used as a stain without eosin to demonstrate connective tissues such as elastic fibers, muscle striations and mucins.

Eosin

Eosin is a fluorescent, xanthene dye which binds to salts with eosinophilic compounds containing positive charges. It is the most suitable stain to combine with an alum hematoxylin to demonstrate the general histological architecture of tissues. Eosin has the ability, with correct differentiation, to distinguish between the cytoplasm of different types of cell, connective tissue fibers and matrices, by staining these differing shades of red and pink.

There are several types of eosin available commercially but Eosin Y is the most widely used and is soluble in water and alcohol. As a cytoplasmic stain, it is usually used as a 0.5 or 1.0% solution in distilled water, with a crystal of thymol added to inhibit the growth of fungi. The addition of a little acetic acid (0.5 ml to 1000 ml stain) is said to sharpen the staining. The majority of the differentiation of eosin staining occurs in the subsequent tap water wash, but a little further occurs during dehydration through the alcohols. The intensity of eosin staining and the degree of differentiation is a matter of individual and laboratory taste. Good film-based photomicrographs of H&E stained tissues are obtained when the eosin staining is relatively intense and the differentiation slight. This is achieved by doubling the routine staining time. However, modern digital cameras can be used with the full range of eosin staining. Alternative red dyes, e.g. phloxine or Biebrich scarlet can be used as an eosin substitute but give a more intense red color to the tissues, and are rarely as amenable to subtle differentiation, making them less effective.

Eosin staining may be intense after mercuric fixation and difficulty may be experienced in obtaining adequate differentiation. Over-differentiation of the eosin, until only the red blood cells and granules of eosinophil polymorphs are stained red, facilitates the location and identification of this type of cell. Combining 10 ml 1% phloxine B, 100 ml 1% eosin Y, 780 ml 95% alcohol and 4 ml glacial acetic acid produces a cytoplasmic stain where muscle is clearly differentiated from collagen and red cells stain bright red.

Hematoxylin

Hematoxylin is extracted from the heartwood ('logwood') of the tree *Hematoxylon campechianum* which originated in the Mexican State of Campeche, but is now mainly cultivated in the West Indies. It is extracted from the logwood with hot water and then precipitated out from the aqueous solution using urea (see prior editions of this text). Hematoxylin itself is not a stain, but hematein, the major oxidization product, is a natural dye responsible for the color properties.

Hematein can be produced from hematoxylin in two ways:

1. *Natural oxidation or 'ripening'* by exposure to light and air. This slow process can take up to 3–4 months, but the resultant solution retains its staining ability for a long time. Ehrlich's and Delafield's hematoxylin solutions are examples of naturally ripened hematoxylins.
2. *Chemical oxidation.* Chemical oxidizing agents convert the hematoxylin to hematein rapidly, so these solutions are ready for use immediately after preparation. Sodium iodate used in Mayer's hematoxylin and mercuric oxide used in Harris's hematoxylin are examples of these agents. These solutions have a shorter useful life than the naturally oxidized hematoxylins because the oxidation process continues in air and light, converting the hematein to a colorless compound.

Hematein is anionic with a poor affinity for tissue and is inadequate as a nuclear stain without the presence of a mordant. The metal cation in the mordant confers a net positive charge to the dye-mordant complex and enables it to bind to anionic tissue sites, e.g. nuclear chromatin. The type of mordant used influences the type of tissue components stained and their final color. Aluminum, iron and tungsten salts are the most useful mordants for hematoxylin, but solutions using lead are used for the demonstration of argyrophil cells.

Most mordants are incorporated into the staining solution, but certain hematoxylins such as Heidenhain's iron require the tissue section to be pre-treated with the mordant before staining.

Hematoxylin solutions are classified according to the mordant used:

- Alum hematoxylins
- Iron hematoxylins
- Tungsten hematoxylins
- Molybdenum hematoxylins
- Lead hematoxylins
- Hematoxylins without mordant.

Alum hematoxylins

These are the most frequently used hematoxylins in the H&E and produce good nuclear staining. The mordant is aluminum, usually aluminum potassium sulfate (potash alum) or aluminum ammonium sulfate (ammonium alum). They stain the nuclei a red color, which is converted to the familiar blue-black when the section is washed in a weak alkali solution. Tap water is usually alkaline enough to produce this color change, but occasionally alkaline solutions such as saturated lithium carbonate, 0.05% ammonia in distilled water, or Scott's tap water substitute are necessary (see Appendix V). This procedure is known as 'blueing'.

The alum hematoxylins can be used either regressively or progressively. In regressive staining the section is over-stained and then differentiated in acid alcohol, followed by blueing. In progressive staining, the section is stained for a predetermined time, staining the nuclei adequately, but leaving the background tissue relatively unstained. The times required for hematoxylin staining with satisfactory differentiation vary according to the type and age of the alum hematoxylin, the tissue type and the personal preference of the pathologist. Ehrlich's, Mayer's, Harris's, Gill's, Cole's and Delafield's are the most commonly used alum hematoxylins for routine H&E staining. Carazzi's hematoxylin is occasionally used, particularly for urgent frozen sections.

Ehrlich's hematoxylin (Ehrlich, 1886)

This naturally ripened alum hematoxylin takes approximately 2 months to ripen but this time can be shortened by placing the unstoppered bottle in a warm sunny position. Once satisfactorily ripened this solution will last in bulk for years and also retains its staining ability in a Coplin jar for several months.

Ehrlich's hematoxylin, as well as being an excellent nuclear stain, also stains mucins including the mucopolysaccharides of cartilage. It is recommended for the staining of bone and cartilage (see Chapter 17).

Preparation of solution

Hematoxylin	2 g
Absolute alcohol	100 ml
Glycerin	100 ml
Distilled water	100 ml
Glacial acetic acid	10 ml
Potassium alum	15 g

The hematoxylin is dissolved in alcohol and the other chemicals are added. Glycerin is added to slow the oxidation process and prolong the shelf life of the stain. Natural ripening in sunlight takes approximately 2 months, but the stain can be chemically ripened if it is needed urgently by adding 50 mg of sodium iodate for every gram of hematoxylin. This will shorten the bench life of the stain. By definition the chemically oxidized variant is not a true Ehrlich's hematoxylin and will not have the same longevity as when naturally oxidized. It should always be filtered before use.

Ehrlich's hematoxylin is a strong solution staining nuclei intensely and crisply. The stain fades more slowly than those stained with other alum hematoxylins. It is suitable for acid-decalcified tissues, and tissues stored in formalin fixatives for long periods of time which become increasingly acidic during the storage period. Ehrlich's hematoxylin is also suitable for tissues which have been fixed in acid fixatives such as Bouin's. Ehrlich's hematoxylin is not ideal for frozen sections.

Delafield's hematoxylin (Delafield & Prudden, 1885)

A naturally ripened alum hematoxylin, Delafield's has similar longevity to Ehrlich's.

Preparation of solution

Hematoxylin	4 g
95% alcohol	125 ml
Saturated aqueous ammonium alum (15 g/100 ml)	400 ml
Glycerin	100 ml

The hematoxylin is dissolved in 25 ml of alcohol, and added to the alum solution. The mixture stands in light and air for 5 days, then filtered. The glycerin is added and a further 100 ml of 95% alcohol. The stain is exposed to light and air for approximately 3–4 months or until it is sufficiently dark in color, then filtered and stored. Filter again before use.

Mayer's hematoxylin (Mayer, 1903)

This hematoxylin is chemically ripened with sodium iodate and is usually used as a regressive stain. It can be useful as a progressive stain, particularly where a nuclear counterstain is required to emphasize the cytoplasmic component demonstrated by a special stain where the acid-alcohol differentiation may destroy or remove the stained cytoplasm. It is used as a nuclear counterstain in many staining methods by applying it for 5–10 minutes until the nuclei are stained, and then 'blued' without any differentiation.

Preparation of solution

Hematoxylin	1 g
Distilled water	1000 ml
Potassium or ammonium alum	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate SLR **or**	50 g
chloral hydrate AR	30 g

The hematoxylin, potassium alum and sodium iodate are dissolved in the distilled water by warming and stirring, or by standing at room temperature overnight. Chloral hydrate and citric acid are added, and the mixture is boiled for 5 minutes, cooled and filtered. If the higher purity chloral hydrate AR grade is used, the amount may be reduced, as shown above. The stain is ready for use immediately. Filter before use.

Harris's hematoxylin (Harris, 1900)

This alum hematoxylin was traditionally ripened with mercuric oxide. Mercury is highly toxic, environmentally unfriendly and has detrimental and corrosive long-term effects on automated staining machines, so sodium or potassium iodate is now generally used for the oxidation. It is a useful general purpose stain giving clear nuclear staining and is particularly valuable as a progressive stain in diagnostic exfoliative cytology.

In routine histological practice it is generally used regressively, but it can be useful when used progressively. When using Harris's hematoxylin as

a progressive stain, an acetic acid-alcohol rinse is a more controllable method for removing excess stain from tissue components. The traditional hydrochloric acid-alcohol acts quickly and indiscriminately and since this is more difficult to control it can result in a light nuclear stain. A 5–10% solution of acetic acid in 70–95% alcohol detaches dye molecules from the cytoplasm and nucleoplasm while keeping nucleic acid complexes intact (Feldman & Dapson, 1985).

Preparation of solution

Hematoxylin	2.5 g
Absolute alcohol	25 ml
Potassium alum	50 g
Distilled water	500 ml
Mercuric oxide **or**	1.25 g
sodium iodate	0.5 g
Glacial acetic acid	20 ml

The hematoxylin is dissolved in the absolute alcohol, and added to the alum which has previously been dissolved in the warm distilled water in a 2 L flask. The mixture is rapidly brought to the boil and the mercuric oxide or sodium iodate is then slowly added. Cool the solution in cold water. When cold, the acetic acid is added and the stain is ready for immediate use. The glacial acetic acid is optional but its inclusion gives more precise and selective staining of nuclei.

Chemically ripened alum hematoxylins lose the quality of the nuclear staining after a few months as a precipitate forms in the stored stain. The stain should be filtered before use, and the staining time may need to be increased. For the best results fresh batches of stain should be prepared every month.

Cole's hematoxylin (Cole, 1943)

This is an alum hematoxylin artificially ripened with alcoholic iodine.

Preparation of solution

Hematoxylin	1.5 g
Saturated aqueous potassium alum	700 ml
1% iodine in 95% alcohol	50 ml
Distilled water	250 ml

The hematoxylin is dissolved in warm distilled water and mixed with the iodine solution. The alum solution is added, and the mixture brought to the boil, then cooled quickly and filtered. The solution is ready for immediate use, but may need filtering after storage, for the same reason as described above for Harris's hematoxylin.

Carazzi's hematoxylin (Carazzi, 1911)

This is an alum hematoxylin which is chemically ripened using potassium iodate.

Preparation of solution

Hematoxylin	5 g
Glycerol	100 ml
Potassium alum	25 g
Distilled water	400 ml
Potassium iodate	0.1 g

The hematoxylin is dissolved in the glycerol, and the alum is dissolved in most of the water overnight. The alum solution is added slowly to the hematoxylin solution, mixing well after each addition. The potassium iodate is dissolved in the rest of the water with gentle warming. It is added to the hematoxylin, alum and glycerol mixture. The final staining solution is mixed well and is then ready for immediate use and remains usable for about 6 months. Care must be taken in preparing the hematoxylin to avoid over oxidation and it is safer if heat is not used to dissolve the reagents. Filter before use.

Like Mayer's hematoxylin, Carazzi's may be used as a progressive nuclear counterstain using a short staining time, followed by 'blueing' in tap water. It is particularly suitable as it is a pale and precise nuclear stain which does not stain any of the cytoplasmic components.

Gill's hematoxylin (Gill et al. 1974, modified)

This is an alum hematoxylin chemically ripened using sodium iodate.

Preparation of solution

Hematoxylin	2 g
Sodium iodate	0.2 g
Aluminum sulfate	17.6 g
Distilled water	750 ml
Ethylene glycol (ethandiol)	250 ml
Glacial acetic acid	20 ml

The distilled water and ethylene glycol are mixed, and the hematoxylin is added. The ethylene glycol is an excellent solvent for hematoxylin as it prevents the formation of surface precipitates (Carson, 1997). Sodium iodate is added for oxidation, and the aluminum sulfate mordant is then added. Finally, the glacial acetic acid is added and the solution is stirred for 1 hour and filtered before use. Carson reported that, although the stain can be used immediately the intensity is improved if allowed to ripen for 1 week in a 37°C incubator. The popularity of Gill's solution has made it one of the more commercially successful formulas.

Double or triple hematoxylin concentrations may be used. These are usually referred to as Gill's I (normal), Gill's II (double), and Gill's III (triple), with the Gill III being the most concentrated. Gill's hematoxylin is the most frequently used for routine H&E staining as it is the most stable and auto oxidation is inhibited with no measurable changes in the solution even after several months. Disadvantages associated with Gill's hematoxylin include staining of the gelatin adhesive, the glass slide itself and some mucus may stain darkly. With Harris's hematoxylin, mucus generally remains unstained, and the glass usually fails to attract the stain. Certain charged sites in the tissue, in the adhesive and on the glass are masked by the Harris mordant, leaving them unavailable for staining. Gill's mordant system fails to do this and the sites attract the dye-mordant complex.

Staining times with alum hematoxylins

The following staining times for alum hematoxylins are only a rough guide because the time needed varies according to the following factors:

- Type of hematoxylin used.
- Age of the stain: as the stain ages the staining time will need to be increased.
- Frequency of use of the stain. A heavily used hematoxylin will lose its staining powers more rapidly and longer staining times will be necessary or, in a frequently used automated staining machine the stain will need to be changed at regular intervals.
- Whether the stain is used progressively or regressively.
- Pre-treatment of tissues or sections, e.g. length of time in fixative or acid decalcifying solution, or whether paraffin or frozen sections.
- Post-treatment of sections, e.g. subsequent acid stains such as van Gieson.
- Personal preference.

The times given in Table 10.1 are a general indication of a suitable range for each type of stain; the optimal time is determined by trial and error. Except where stated, these figures refer to normally fixed paraffin sections. As a rule, the time should be considerably shortened for frozen sections and increased for decalcified tissues and those stored for a long time in non-buffered formalin.

Disadvantages of alum hematoxylins

The major disadvantage of alum hematoxylin stains is their sensitivity to any subsequently applied acidic staining solutions, e.g. van Gieson and trichrome stains. The application of the picric acid-acid fuchsin mixture in van Gieson's stain removes most of the hematoxylin so that the nuclei are barely discernible. Satisfactory nuclear staining is achieved, in this case, by using an iron-mordanted hematoxylin such as Weigert's hematoxylin (see p. 132), which is more resistant to the effect of picric acid. A suitable alternative is the combination of a celestine blue staining solution with an alum hematoxylin. Celestine blue

Table 10.1 Staining times with alum hematoxylins

Cole's	20–45 min
Delafield's	15–20 min
Ehrlich's progressive	20–45 min
Mayer's progressive	10–20 min
Mayer's regressive	5–10 min
Harris's progressive in cytology	4–30 s
Harris's regressive	5–15 min
Carazzi's progressive	1–2 min
Carazzi's regressive	45 s
Carazzi's with frozen sections, see text	1 min
Gill's I regressive	5–15 min

is resistant to the effects of acid, and the ferric salt in the prepared celestine blue solution strengthens the bond between the nucleus and the alum hematoxylin providing a strong nuclear stain more resistant to acid.

Celestine blue-alum hematoxylin procedure

Celestine blue solution

Celestine blue B	2.5 g
Ferric ammonium sulfate	25 g
Glycerin	70 ml
Distilled water	500 ml

The ferric ammonium sulfate is dissolved in cold distilled water with stirring, the celestine blue is added and the mixture is boiled for a few minutes. After cooling, the stain is filtered and glycerin is added. The final stain should be usable for over 5 months. Filter before use.

Method

1. Dewax sections, rehydrate through descending grades of alcohol and take to water.
2. Stain in celestine blue solution for 5 minutes.
3. Rinse in distilled water.
4. Stain in an alum hematoxylin, e.g. Mayer's or Cole's for 5 minutes.
5. Wash in water until blue.
6. Proceed with required staining technique.

Routine staining procedures using alum hematoxylins

Non-automated hematoxylin and eosin stain for paraffin sections

Method

1. Dewax sections, rehydrate through descending grades of alcohol to water.
2. Remove fixation pigments if necessary.
3. Stain in an alum hematoxylin of choice for a suitable time.
4. Wash well in running tap water until sections 'blue' for 5 minutes or less.
5. Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5–10 seconds.
6. Wash well in tap water until sections are again 'blue' (10–15 minutes).
7. **Or** blue by dipping in an alkaline solution followed by a 5 minute tap water wash.
8. Stain in 1% eosin Y for 10 minutes.
9. Wash in running tap water for 1–5 minutes.
10. Dehydrate through alcohols, clear, and mount.

Results

Nuclei	blue/black
Cytoplasm	varying shades of pink
Muscle fibers	deep pink/red
Red blood cells	orange/red
Fibrin	deep pink

Notes

The structures and substances other than nuclei may be hematoxyphilic to varying degrees, e.g. fungal hyphae and calcium deposits are often stained deep blue-black.

Rapid hematoxylin and eosin stain for urgent frozen sections

Method

1. Freeze suitable tissue block onto a chuck.
2. Cut cryostat sections at 3–6 µm thickness.
3. Fix section in 10% neutral buffered formalin at room temperature for 20 seconds.
4. Rinse in tap water.
5. Stain in double strength Carazzi's hematoxylin for 1 minute.
6. Wash well in tap water for 10–20 seconds.
7. Stain in 1% aqueous eosin for 10 seconds.
8. Rinse in tap water.
9. Dehydrate, clear and mount.

Papanicolaou staining method for cervical cytological preparations

Combining Harris's hematoxylin as the optimal nuclear stain with Orange G 6 (OG 6) and EA 50 in the Papanicolaou stain for cervical cytology produces the subtle range of green, blue, and pink hues to the cell cytoplasm.

Most laboratories use commercial stains titrated for a specific automated staining machine or regime, the results must retain the transparent quality of the

cytoplasmic stain and the nuclear chromatin should be easily distinguished.

Papanicolaou method (Papanicolaou, 1942)

Solutions for Papanicolaou staining

Harris's hematoxylin

Orange G 6 solution (OG 6)

10% aqueous Orange G	50 ml
Alcohol	950 ml
Phosphotungstic acid	0–15 g

EA 50 solution

0.04 M light green SF	10 ml
0.3 M eosin Y	20 ml
Phosphotungstic acid	2 g
Alcohol	750 ml
Methanol	250 ml
Glacial acetic acid	20 ml

Filter all stains before use.

Method

1. Remove polyethylene glycol fixative in 50% alcohol for 2 minutes.
2. Hydrate in 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
3. Rinse in water for 1 minute.
4. Stain in Harris's hematoxylin for 5 minutes.
5. Rinse in water for 2 minutes.
6. Differentiate in 0.5% aqueous hydrochloric acid for 10 seconds approx.
7. Rinse in water for 2 minutes.
8. 'Blue' in Scott's tap water substitute for 2 minutes.
9. Rinse in water for 2 minutes.
10. Dehydrate, 70% alcohol for 2 minutes.
11. Dehydrate, 95% alcohol for 2 minutes.
12. Dehydrate in fresh 95% alcohol for 2 minutes.
13. Stain in OG 6 for 2 minutes.
14. Rinse in 95% alcohol for 2 minutes.
15. Rinse in fresh 95% alcohol for 2 minutes.
16. Stain in EA 50 for 3 minutes.
17. Rinse in 95% alcohol for 1 minute.
18. Dehydrate, clear and mount.

The staining times are adjusted to suit personal preference for a darker or paler stain. Alternatives to Scott's tap water substitute include 0.1% ammoniated water or a weak aqueous solution of lithium carbonate.

Results

Nuclei	blue/black
Cytoplasm (non-keratinizing squamous cells)	blue/green
Keratinizing cells	pink/orange

Note

Change stains frequently.

Iron hematoxylins

These hematoxylin solutions use iron salts, usually ferric chloride and ferric ammonium sulfate, both as the oxidizing agent and the mordant. The most common iron hematoxylins are:

- Weigert's
- Heidenhain's
- Loyez for myelin
- Verhöeff's for elastin fibers.

Over-oxidation of the hematoxylin is a problem with these stains, so either prepare separate mordant/oxidant and hematoxylin solutions then mix immediately before use, e.g. in Weigert's or use them consecutively, e.g. Heidenhain's and Loyez. The iron salt content produces a solution with a strong oxidizing ability and this allows it to be used as a subsequent differentiating solution after the hematoxylin, as well as the mordant before the dye.

The iron hematoxylins are capable of demonstrating a much wider range of tissue structures than the alum hematoxylins, but the techniques are more time consuming, and usually incorporate a differentiation stage which needs microscopic control for accuracy. The use of iron hematoxylin based methods for the specific identification of phospholipids is briefly discussed in Appendix I.

Weigert's hematoxylin

This is an iron hematoxylin in which ferric chloride is used as the mordant/oxidant. The iron and hematoxylin solutions are prepared separately and are mixed immediately before use.

Weigert's hematoxylin (Weigert, 1904)

Preparation of solutions

Hematoxylin solution

Hematoxylin	1 g
Absolute alcohol	100 ml

This is allowed to ripen naturally for 4 weeks before use.

Iron solution

30% aqueous ferric chloride (anhydrous)	4 ml
Hydrochloric acid (concentrated)	1 ml
Distilled water	95 ml

The iron solution is filtered and added to an equal volume of the hematoxylin solution immediately before use. The mixture should be a violet-black color and discarded if it is brown. It is used as a nuclear stain in techniques where acidic staining solutions are to be applied to the sections subsequently, e.g. van Gieson stain. A staining time of 15–30 minutes is usual. In this role, Weigert's hematoxylin has been largely replaced by the more convenient celestine blue-alum hematoxylin procedure. It does remain a useful stain with eosin for CNS tissues. For the purist who prefers a black nuclear counterstain with a van Gieson technique, the ferrous hematein technique of Slidders (1969) is satisfactory.

Heidenhain's hematoxylin

This iron hematoxylin uses ferric ammonium sulfate as the oxidant/mordant and the differentiating fluid. The iron solution is used first, the section is then treated with the hematoxylin solution until it is over-stained and it is then differentiated with the iron solution under microscopic control.

Heidenhain's hematoxylin can be used to demonstrate many structures according to the degree of differentiation. After initial staining, all tissue components are black or dark gray-black. The hematoxylin staining is removed progressively from different tissue structures at different rates using the iron alum solution. Mitochondria, muscle striations, nuclear chromatin, and myelin are demonstrated. The black color disappears first from mitochondria, then from muscle striations and finally from nuclear chromatin. Longer differentiation will remove the stain from almost all structures, although red blood cells and keratin retain the stain the longest. More easily controllable differentiation can be achieved if the differentiating iron alum solution is diluted with an equal volume of distilled water or an alcoholic picric acid solution.

Heidenhain's hematoxylin (Heidenhain, 1896)

Preparation of solutions

Hematoxylin solution

Hematoxylin	0.5 g
Absolute alcohol	10 ml
Distilled water	90 ml

The hematoxylin is dissolved in the alcohol, and the water is then added. The solution is allowed to ripen naturally for 4 weeks before use.

Iron solution (5% iron alum)

Ferric ammonium sulfate	5 g
Distilled water	100 ml

It is important that only the clear violet crystals of ferric ammonium sulfate be used.

Method

1. Dewax sections, rehydrate through descending grades of alcohol to water.
2. Mordant in iron solution (5% iron alum) for 1 hour (see note a).
3. Rinse in distilled water.
4. Stain in Heidenhain's hematoxylin solution for 1 hour (see note a).
5. Wash in running tap water.
6. Differentiate in the iron solution, or the iron solution diluted with an equal volume of distilled water. Alternate a rinse in differentiator with a rinse in tap water. The degree of differentiation is controlled microscopically until the desired structure is clearly demonstrated (see note b).
7. Wash in running tap water for 10 minutes.
8. Dehydrate, clear, and mount.

Results

Mitochondria, muscle striations, myelin, chromatin etc. are gray-black.

Notes

a. The time needed in the mordant and stain will vary according to the fixative used; usually 1 hour in

each solution is satisfactory, but tissues fixed in dichromate solutions need longer. The following times are a rough guide. Tissues fixed in formalin solutions, formal sublimate, Susa, Bouin's, and Carnoy's, 1 hour. Tissues fixed in Helly's or Zenker's, 3 hours. Tissues fixed in osmium tetroxide and Fleming's fluid, up to 24 hours.

b. Differentiation is difficult to judge, the slide is dipped in and out of the 5% iron alum until the background of the slide is clear, then checked microscopically. If the differentiation proceeds beyond the desired end, the section can be restained for the same time in the hematoxylin solution and differentiation attempted again.

c. Cytoplasmic counterstains, e.g. aqueous eosin or Orange G are rarely necessary although they may be used to accentuate nuclear chromatin, particularly for the demonstration of chromosomes or mitoses.

d. Sections stained with Heidenhain's iron hematoxylin are resistant to fading if the section is washed well after the differentiation stage to remove all traces of iron alum.

Loyez hematoxylin (Loyez, 1910)

This iron hematoxylin uses ferric ammonium sulfate as the mordant. The mordant and hematoxylin solutions are used consecutively, and differentiation is by Weigert's differentiator (4 g borax, and 5 g potassium ferricyanide dissolved in 200 ml distilled water). It is used to demonstrate myelin and can be applied to paraffin, frozen or nitrocellulose sections. There are two shorter methods similar to that of Loyez. The Heidenhain myelin stain (not to be confused with Heidenhain's iron hematoxylin) is essentially the Loyez technique but judicious selection of staining time removes the need for separate differentiation. The second variant is the short Weil technique in which the mordant and dye are mixed before use rather than used consecutively.

Verhöeff's hematoxylin (Verhöeff, 1908)

This iron hematoxylin is used to demonstrate elastic fibers. Ferric chloride and Lugol's iodine are included in the hematoxylin staining solution and 2% aqueous ferric chloride is used as the differentiator. The coarse elastic fibers in the tissue are stained black.

Other elastin staining methods may be used to demonstrate finer fibers but, for the high contrast required for photomicrography, the intense black staining produced by Verhöeff's is ideal. It is also used to stain elastin fibers as part of Movat's pentachrome.

Tungsten hematoxylins

There is only one widely used tungsten hematoxylin, although there are many variants on the original Mallory phosphotungstic acid hematoxylin (PTAH) technique. Mallory (1897, 1900) combined hematoxylin with 1% aqueous phosphotungstic acid, the latter acting as the mordant. It is possible to prepare a staining solution using hematein instead of hematoxylin. Here, the oxidation process is unnecessary and the staining solution can be used immediately, but its activity is comparatively short lived. The hematoxylin can be oxidized chemically by using a potassium permanganate solution and the solution is usable within 24 hours. The most satisfactory, but time consuming, method of preparation is to allow natural ripening of the tungsten hematoxylin solution in light and air. The PTAH solution produced may take some months to ripen, but it will remain usable for many years. Its use is applicable to both CNS material and general tissue structure, and tissues fixed in any of the standard fixatives. Staining times will vary according to the method of preparation, the fixative used, and the tissue structure to be demonstrated. Staining is more precise after the section has been treated with an acid dichromate solution, and after a Mallory bleach procedure.

PTAH staining technique solution using hematein (Shum & Hon, 1969)

Preparation of solutions

Solution a

Hematein	0.8 g
Distilled water	1 ml

Grind the 0.8 g hematein to a paste with the distilled water. The paste should be chocolate brown, lighter colors are usually indicative of an unsuitable batch of hematein and should be discarded.

Solution b

Phosphotungstic acid	0.9 g
Distilled water	9 ml

Mix solution **a** and solution **b**, bring to the boil, then cool and filter.

Method

1. Dewax, rehydrate through descending grades of alcohol to water.
2. Treat with acid permanganate (see below) for 5 minutes.
3. Rinse in tap water.
4. Bleach with 5% aqueous oxalic acid.
5. Wash well in tap water.
6. Stain in PTAH solution for 12–24 hours at room temperature.
7. Wash in distilled water.
8. Dehydrate rapidly, clear and mount.

The solution may be used at 56°C for several hours, but staining for a longer time at room temperature is preferable as the results are more precise.

Mallory's PTAH technique (Mallory, 1900)

Preparation of solutions

PTAH staining solution, chemically oxidized with potassium permanganate

Hematoxylin	0.5 g
Phosphotungstic acid	10 g
Distilled water	500 ml
0.25% aqueous potassium permanganate	25 ml

Hematoxylin is dissolved in 100 ml of the distilled water, and the phosphotungstic acid in 400 ml. The two solutions are mixed and the potassium permanganate solution is added. The stain can be used next day, but peak staining activity is reached after 7 days. Continuing oxidation of the hematoxylin means that this stain has a comparatively short life.

PTAH staining solution, naturally oxidized

Hematoxylin	0.5 g
Phosphotungstic acid	5 g
Distilled water	500 ml

The solids are dissolved in the distilled water and mixed as in the recipe above. The stain is allowed to ripen naturally in a loosely stoppered bottle in a warm, light place for several months.

Acid dichromate solution

10% HCl in absolute alcohol	12 ml
3% aqueous potassium dichromate	36 ml

Acid permanganate solution

0.5% aqueous potassium permanganate	50 ml
3% sulfuric acid	2.5 ml

Method

1. Dewax, rehydrate through descending grades of alcohol to water.
2. Place in acid dichromate solution for 30 minutes.
3. Wash in tap water.
4. Treat with acid permanganate solution for 1 minute.
5. Wash in tap water.
6. Bleach in 1% oxalic acid.
7. Rinse in tap water.
8. Stain in either of the Mallory's PTAH stains overnight.
9. Dehydrate through ascending grades of alcohol, clear, and mount.

Results

Muscle striations, neuroglia, fibers, fibrin and amebae	dark blue
Nuclei, cilia, red blood cells	blue
Myelin	lighter blue
Collagen, osteoid, cartilage, elastic fibers	deep brownish-red
Cytoplasm	pale pinkish-brown

Notes

a. The acid dichromate treatment (post chroming) can be omitted if fixation has been by a chromate-containing fixative.

b. Dehydration should be rapid since water and alcohol may remove some of the stain. If the sections are too blue, a degree of differentiation can be achieved during dehydration. Dehydration may be commenced in 95% alcohol, and CNS sections may need thorough washing in 95% alcohol for several minutes to remove excess red stain.

c. The times in the dichromate, permanganate and stain may need to be modified depending on the nature of the tissue and the feature to be demonstrated.

Molybdenum hematoxylins

Hematoxylin solutions which use molybdic acid as the mordant are rare and the only technique which gained any acceptance was the Thomas (1941) technique mentioned by McManus and Mowry (1964). They recommend the method for the demonstration of collagen and coarse reticulin, although more valuable and widely accepted techniques for these connective tissue fibers exist. The Thomas method also stains argentaffin cell granules.

Phosphomolybdic acid hematoxylin stain (Thomas, 1941)

Preparation of solutions

Hematoxylin solution

Hematoxylin	2.5 g
Dioxane	49 ml
Hydrogen peroxide	1 ml

Phosphomolybdic acid solution

Phosphomolybdic acid	16.5 g
Distilled water	44 ml
Diethylene glycol	11 ml

The phosphomolybdic acid solution is filtered and 50 ml of the filtrate is added to the hematoxylin solution. The resultant solution, which should be dark violet in color, is allowed to stand for 24 hours before use.

Method

1. Dewax, rehydrate through descending grades of alcohol to water.
2. Stain with phosphomolybdic acid hematoxylin for 2 minutes.
3. Wash with distilled water.
4. Drop picro-acetic alcohol (see note a) onto section, then wash away immediately with distilled water.
5. Rinse in tap water, dehydrate through 95% and absolute alcohol, clear, and mount.

Results

Collagen and coarse reticulin	violet to black
Argentaffin cells	black
Nuclei	pale blue
Paneth cells	orange

Notes

a. Picro-acetic alcohol (picric acid 0.5 g, glacial acetic acid 0.5 ml, 70% alcohol 100 ml) acts as a differentiator but this stage can be omitted.

b. Tissues fixed in dichromate do not give good results.

Lead hematoxylins

Hematoxylin solutions which incorporate lead salts have been used in the demonstration of the granules in the endocrine cells of the alimentary tract and other regions. The most practical diagnostic application is in the identification of endocrine cells in some tumors, but it is also used in research procedures, e.g. in the localization of gastrin-secreting cells in the stomach (Beltrami et al., 1975). Their use has largely been superseded by immunohistochemistry.

Hematoxylin without a mordant

Freshly prepared hematoxylin solutions used without a mordant have been used to demonstrate various minerals in tissue sections. Mallory (1938) described a method for lead, and later published a similar method capable of demonstrating iron and copper (Mallory & Parker, 1939).

The uses of hematoxylin stains are briefly summarized in Table 10.2.

Quality control in routine H&E staining

The majority of diagnoses are made by a pathologist examining H&E stained paraffin sections produced in bulk by an automated staining machine. The consistency of staining is vital to avoid difficult histological interpretation.

In general, automated staining machines allow accurate and consistent staining, differentiation and dehydration by adjusting the times of each step (see Chapter 11). However, variability in the staining solutions used may necessitate adjustment of the staining times. These problems are usually associated with the hematoxylin rather than eosin. Common variables with commercially produced hematoxylin are a variation in batch number, a change of supplier and

Table 10.2 The uses of hematoxylin stains

Hematoxylin	Applications	Oxidant	Mordant
Ehrlich	Nuclear stain used with eosin and stains some mucins	Natural	Alum
Delafield	Nuclear stain used with eosin	Natural	Alum
Mayer	Nuclear stain used with eosin and nuclear counterstain	Sodium iodide	Alum
Harris	Nuclear stain used with eosin	Mercuric oxide	Alum
Cole	Nuclear stain used with eosin	Iodine	Alum
Carazzi	Nuclear stain used with eosin in frozen sections	Potassium iodate	Alum
Gill	Nuclear stain used with eosin	Sodium iodate	Alum
Weigert	Nuclear stain used with acid dyes	Natural	Iron
Heidenhain	Intranuclear detail, muscle striations	Natural	Iron
Verhöeff	Elastic fibers	Natural	Iron
Loyez	Myelin	Natural	Iron
Mallory PTAH	Fibrin, muscle striations, glial fibers	Natural	Tungsten
Thomas	Collagen, endocrine cell granules	Hydrogen peroxide	Molybdenum
Solcia	Endocrine cell granules	No oxidant	Lead
Mallory	Iron, copper, lead	No oxidant	No mordant
Weigert-Pal	Myelin in block preparation	No oxidant	Chromium-copper

pH differences. Laboratory produced hematoxylin may have different staining properties each time it is prepared, despite the same preparation instructions being employed. Aside from individual variability and those between personnel following the same 'recipe', the age of the stain and the degree of its usage will also affect the staining properties. New batches of stain must be checked for efficacy against current or earlier batches, and staining times must be adjusted to give uniformity. It is also important to realize that other factors, e.g. fixation, variations in processing schedules, section thickness and excessive hot plate temperatures, may all lead to a variation in staining.

Difficult sections

The problem of using hematoxylin as a nuclear counterstain when other acidic dyes are being used, e.g. van Gieson has already been mentioned. A similar problem occurs when attempting to stain paraffin sections which have been fixed for a long time in a formalin fixative which has gradually become more acidic. This problem may be compounded in tissues and/or paraffin wax blocks sent from countries with hot climates because the tissue may be fixed in a poor quality, unbuffered, non-neutral formalin fixative which deteriorates more rapidly in the heat. Adequate nuclear staining with hematoxylin without some staining of the cytoplasm is difficult in this situation. It often results in a uniformly muddy purple to the finished section after eosin has been applied. There are two main ways in which diagnostically acceptable H&E sections can be obtained in these circumstances. One is the use of the celestine blue-alum hematoxylin sequence, and the other is the use of an iron hematoxylin such as Weigert's.

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Automation in the histology department

11

Greg Zardin • Lynne Braithwaite

Introduction

Transforming a tissue specimen from fixed material to stained sections is a multiple step process which began as separate manual tasks. Indeed, histology in the last century has been the slowest of the laboratory medicine departments to innovate and keep pace with the speed required for a modern dynamic hospital. Whereas the availability of high-throughput analyzers has made same-day results the expected norm in the blood sciences, histology, with its labor-intensive preparations and processes usually sees result turnaround time (TAT) for biopsies and surgical samples being counted in days or weeks rather than hours or minutes.

Historically the lengthy TATs of the histopathology laboratory have been unavoidable due to the technical requirements and the multiple manual stages involved from tissue handling through to the preparation of slides. However, alongside the demands of modern medicine many laboratories have introduced new semi and fully automated processes and tracking systems designed to enable rapid, accurate and safe histopathology reporting.

The modern manipulations to deliver these tissue handling steps are covered in the various chapters of this book, but increasingly this is an automated and standardized reality. Histology automation is perceived as a relatively recent movement, but examples began to be seen as far back as 1945 (Titford, 2006). This chapter will deal with various components of laboratory activity and the automation which currently exists. Whilst there are many companies and systems to facilitate these automated solutions to histology laboratory practice, only some illustrative examples are given and the discussion cannot be all-encompassing. Indeed, one must look at this evolving technology arena regularly in order to to keep up to date with the companies which serve this aspect of laboratory practice and their equipment.

The drivers for change

Drivers for the automation of histology processing are various, but principally hinge on two elements: financial budgets (generally constrained) and the need for rapid sample analysis (patient and clinician led). The increase and availability of preventative medicine such as screening protocols, and the development of specific testing for personalized medicine within an aging population, have all contributed to an increase in histology workload. Globally, laboratories are expected to be more efficient than ever. The trend being seen in terms of economies of scale and diagnostic national and international guidelines has pushed towards a minimum number of samples for laboratory efficiency. There are fewer, larger laboratories processing thousands of specimens per month.

The changing nature of laboratory accreditation is another driver to the introduction of automation. The required standards for an accredited laboratory have been expanded to include the validation and verification of all processes, as well as standardizing the equipment and reagents used. The innate production of this type of process normalization and audit information is one of the strengths of an automated procedure.

Technology now exists which, combined with adaptations to work practices, allows results to be available within 24 hours of a biopsy being taken for small and straightforward samples. This should lead

to an improved clinical response and patient outcome without an increase in cost. The goal of introducing a more automated process into histology is to enable a leaner, more efficient process which benefits staff, patients and the service user/s.

Finally there are subsidiary drivers to be considered. For example, looking at processing, one can appreciate that tissue samples needing to move from fixative to paraffin wax requires transfer between multiple solvents and impregnation periods at each stage; this normally takes several hours. Automation of this stage reduces the requirement for manual intervention and allows the process to occur faster or overnight, assisting TATs. Other beneficial outcomes to these systems include the increased safety of the user as the process occurs in an enclosed environment with minimal reagent handling. This has significant health and safety benefits.

Barcode technology and automated sample tracking

A laboratory's responsibility for a specimen (and the laboratory's TAT) begins as soon as the tissue has been removed from the patient. It is from here automation and tracking can begin. Systems such as the Menerini Tissue SAFE are available. These record the time the specimen was taken, when formaldehyde was added and the temperature at which the specimen was transported to the laboratory, therefore maintaining standardization. This allows comprehensive verifiable data on the pre-analysis handling of the specimen (Menerini, 2016).

Sample tracking continues once the specimen is received and passes through all the laboratory processes, ultimately producing a diagnostic report and recording both the disposal of excess material, and specimen storage. The production of an audit trail and the associated chain of custody can be a labor intensive process both to complete and interrogate. Indeed, this mundane task of the completion of an audit trail can account for a significant portion of the departmental workload, the majority of which is the responsibility of the laboratory staff.

In many laboratories the creation of this audit trail has been constructed piecemeal over a period of time, with each step often recorded in a different format and the records often stored in separate physical locations within the laboratory. As a result, laboratories have a plethora of different systems in use including (but not limited to) colored slides and beads, lists of specimens, log books, worksheets and initials here, there and everywhere! This approach arises as a result of the different requirements for the histology material as the specimen moves through the histology process. It is not easy to capture all the required information in a centralized location and there has been a lack of computer software designed specifically for this task.

A comprehensive computer system which records key aspects of the process, such as who has booked in, dissected, embedded, trimmed, cut and checked a specimen can be a useful information management tool.

Barcoding specimens on receipt and integrating this into the laboratory information management system (LIMS) can help facilitate tracking through the laboratory processes. Printing corresponding barcodes onto all of the forms, blocks and slides associated with the specimen and scanning at every station in the laboratory process can provide a comprehensive tracking system from the time a specimen enters the laboratory, right up to the time the sample analysis is concluded. Using purpose-built software with user identification, this kind of minute-by-minute, station-to-station tracking can also provide a detailed record of the personnel involved at each step, detailed data on which equipment was used (where applicable), and the overall laboratory efficiency.

Not only does this automation create the audit trail with a fraction of the user time currently required, it also integrates this information and displays it in an easy-to-use dashboard format. This information can easily and quickly be interrogated to provide workflow, error identification and quality indication information. This is simply not possible with conventional manual systems.

The other advantage of barcode technology is that it enables the automation of the transcription of information from one medium to another. For example, slides can be automatically printed with the laboratory accession number, patient name and

stain generated by the LIMS system and driven directly from the block barcode. This makes the process much faster, safer and more reliable than error-prone manual transcription by humans. At the same time the audit trail attached to the block can be automatically updated with details such as the microtome used and microtomist performing the task.

Many laboratories currently manually transcribe tens of thousands of digits per day and the potential adverse impact of any transcription error necessitates several checks to ensure that the manual transcription of information is correct. The appeal of automating this step wherever possible can be readily appreciated. Providers claim that the correct use of a proprietary system of this kind can reduce the chance of transcription error to almost zero (Roche, 2011).

Implementation of these error reducing automated steps can significantly minimize the risk of adverse events reducing reliance on vigilance at each process step. This permits streaming of the complex array of double and triple checks, thereby significantly improving productivity. There is also an improvement in the ability to access relevant information at each point in the workflow from block or slide.

Automated sample transcription and tracking can either be achieved via in-house development or purchased as whole tracking systems, such as the Leica CEREBRO system, Dako's True Positive ID or VENTANA/Roche's VANTAGE. Some of the advantages and disadvantages associated with each method are outlined in Table 11.1.

Dissection/grossing

Dissection remains a hands-on area of the laboratory with knowledge and training the main requirements for best practice. However, the implementation of macroscopic photography and videography equipment can assist in both of these aspects.

Photographs can be used to store images of forms, blocks and pot labeling and other relevant information e.g. where certain blocks have been taken from a macro specimen. These images can be stored and annotated, improving the record of the macroscopy and dissection of complex specimens. The images can then be made available to the pathologist at the time of reporting and during future discussions, e.g. multidisciplinary team (MDT) meetings.

Table 11.1 The advantages and disadvantages of in-house and commercially available specimen tracking systems

In-house	Commercial
Cheaper initial costs	Requires additional funding
Flexible	Service contract provided
Bespoke solution	Single joined-up solution
Expert support in-house	On-going development and software updates
Asset creation	Try before buy
Requires in-house skills	Expensive, but costs may be offset with other service contract commitments
Difficult to join up components	Tied in to single provider
Requires time to develop	Closed system – requires engineer response
Requires IT resource commitment	Ongoing support costs
Works around your departmental needs	May require adaption of whole laboratory process to correlate with system upon implementation

Their use allows dissection images to be taken which can be used as training tools, to write standard operating procedures (SOP's) in a video format, or to live stream an unusual case for pathologists to discuss. Their use can also allow for remote pathology, with real-time guidance given to the dissector by off-site pathologists viewing the dissection over a video link or web-based systems.

Many systems now exist which fulfill this role and can be permanently mounted over the dissection table. Examples currently include the Menerini MACROpath and Cirdan's Pathlite Macro Camera Station.

Processing

To take a tissue specimen from fixative to paraffin wax requires transfer between multiple solvents and impregnation periods, often taking many hours. This is one of the most commonly automated stages in any histology laboratory, reducing the requirement for manual intervention and allows the process to occur overnight. The length of processing schedules can be reduced with automation, as it becomes possible to use heat and vacuum-assisted impregnation techniques. Other beneficial outcomes to these systems include the increased safety of the user as a result of the process occurring in an enclosed environment, with minimal reagent handling.

Specimen processing was the original automated procedure in histology and remains possibly the most widespread example of histology automation today. The first examples of automated processing in histology involved a dish into which samples were placed. This then revolved through the processing chemicals; a predecessor to the current carousel type tissue processors.

The majority are now stationary chamber processors, where tissue samples are placed into a retort into which the reagents are pumped in and drained out according to a processing schedule. Early advances on this design resulted in the option to use a vacuum and/or convection style heat exchange to encourage rapid solution permeation and hence improved, faster, more reliable tissue processing. With conventional processors of this type, smaller tissue samples can be processed in under 2 hours, allowing them to be run continuously throughout the day. To ensure adequate processing, however, the majority of blocks are still restricted to a processing schedule in excess of 8 hours. In practice this means overnight processing of larger tissue blocks, leads to a minimum of 1 day delay in slide production and the batching of the majority of the following day's workload.

Shortening the processing procedure has been successfully achieved by replacing or enhancing the convection heating methods with microwave heating. This shortens the processing schedule and can remove the need for hazardous chemicals such as xylene and, in some cases, formalin. Despite the proven benefits, such as the production of high quality staining and reduced tissue shrinkage, with shorter process times and reduced reagent costs over conventional methods, microwave processing has been slow to catch on (Metgud et al., 2013). This may partly be due to concerns to antigen preservation as a result of the irregular distribution of energy due to reflection and interference within the chamber. Many systems incorporate a platform or other device to facilitate uniform heat exposure. Two examples of processors which can use microwave technology to reduce processing times are the Leica PELORIS and the Sakura Finetek Tissue-Tek® Xpress®.

The Tissue-Tek® Xpress® (Fig. 11.1) is a microwave unit with reaction chambers designed for uniform distribution of electromagnetic waves to uniformly heat the samples. All samples must be cut to a uniform thickness of no more than 2 mm which allows both biopsies and larger specimens to be processed on the same run. Up to 40 blocks can be added to the processor every 20 minutes in a continuous process, which promotes LEAN workflow by eliminating batching (Sakura Finetek, 2014).

Reagents must be purchased ready-to-use, and the system is xylene and formalin free. A pre-processing step is required, consisting of fixation with Tissue-Tek® Xpress® Molecular Fixative to ensure the preservation of DNA, RNA and proteins in the paraffin wax block.

The Leica PELORIS (Fig. 11.2) is a dual retort processor capable of traditional or xylene-free processing. Xylene-free protocols use two sets of

Fig. 11.1 Sakura Finetec Tissue-Tek® Xpress®.

dehydrants: industrial denatured alcohol (IDA) and isopropyl alcohol (IPA) rather than separate dehydration and clearing steps. This allows the use of higher temperature paraffin wax impregnation steps leading to faster processing.

Each retort can be run separately and hold 300 cassettes across 3 baskets. This scheduling flexibility together with faster xylene-free programming can aid in a laboratory's LEAN workflow. One-, two- and four-hour programs allow almost all diagnostic biopsies and many other small specimens within a department to be processed through the day, greatly improving turnaround times, and leading to a continual flow process. As a result, small biopsies can be processed during the day, and as the embedding of blocks processed overnight finishes, these rapid runs become available for embedding and sectioning. Staggering the loading of each retort throughout the day ensures continuous flow.

The Leica PELORIS also features automated reagent management, monitoring reagent usage and alerting the user when reagent purity has dropped below a pre-set prescribed limit. This ensures that processing quality is maintained at an acceptable standard and reduces reagent usage and associated costs (Leica Biosystems, 2016).

Fig. 11.2 Leica Biosystems PELORIS.

Recent trends in enclosed, automated processor design show the increased awareness and the responsibility that the laboratory has to the health and safety of their staff. These concerns have led to a new generation of processors which have minimized hazardous reagent handling. Processors such as the Thermo Fisher EXCELSIOR and Milestone LOGOS are designed to accept the original reagent transport containers which are stored in the base of the machine. This removes the need to transfer reagents between containers, minimizing operator exposure to the reagents and also reducing reagent change times.

Embedding

This has always been the first task at the start of the day for the laboratory staff. Even with continuous processing, all blocks requiring an extended

Fig. 11.3 Sakura Finetek Paraform cassette.

processing schedule tend to become available to embed as a single large batch at the beginning of the day, and in some departments the embedding of this batch may take the entire day.

Automated embedding equipment now exists which involves orienting the tissue at dissection and processing it in the mold/capsule it will be embedded in. This removes any requirement for orientation at the embedding stage and enables this step to be automated, freeing up the personnel to do other tasks. Two examples of automated embedding equipment are the Sakura Finetek Tissue-Tek® AutoTEC® and Milestone's SYNERGY system.

The Sakura Finetek system, the Tissue-Tek® AutoTEC® uses Paraform cassettes which hold the orientation of tissue placed into them at dissection. The auto-embedder then embeds the entire Paraform cassette holding the tissue in the retained orientation (Fig. 11.3).

The Paraform cassettes' plastic mesh is the same density as the solid paraffin so the resulting block can be sectioned using routine microtomy techniques. The system is capable of embedding up to 120 cassettes per hour with continuous loading of 4 magazines (Sakura Finetek, 2016).

Milestone has introduced their SYNERGY system which they describe as an all-in-one processing and embedding method. This involves placing the tissue into disposable molds prior to processing which are then kept in place with sponge and the printed cassette. Post-processing the mold/cassette unit is transferred to a cold plate to set, after which the blocks can be removed for microtomy.

Auto-embedding technology is still in its infancy, and there are currently several limitations to its use. When looking to purchase an auto-embedder, careful consideration must be given to the ongoing cost of the product and its required consumables, balanced against the cost of the laboratory personnel it may replace. As the precision of the tissue orientation with automated systems is currently poor, thought must be given to the proportion of the laboratory workload for which it which would be appropriate. For example, it would not be possible to continue to orient GI biopsies, which some laboratories routinely position to ensure mucosal cross-section.

With this in mind, the question to address is whether the auto-embedder may deal with sufficient embedding to significantly improve the work-flow and free up sufficient personnel to make an advantageous change to the TATs. Finally, in this arena, one has to address the issue of what back-up will exist if the auto-embedder is broken.

Trimming and microtomy

Microtomy is another area where full automation is yet to be realized, as it depends on the judgement of the microtomist to both trim the tissue to the required depth, and determine when an adequate section has been produced. This is not to say that the process cannot be improved by the introduction of automated ancillary elements. As discussed briefly above workflow, accountability and safety in this area all have the potential to be significantly improved with the use of barcoded cassettes producing labels or printed slides at the microtomy station (Fig. 11.4).

Manual transcription of numbers from block to slide is still a common sight in the majority of laboratories, despite significant opportunities for error. As workloads increase, double or triple checks became required to detect these potential errors and the quality assurance process becomes time consuming.

The use of a barcoded cassette, barcode reader and slide printer at each microtome station removes the need for many of these QA steps by automatically producing a barcoded slide or slide label at the point of production. Done correctly this results in a reduction in human transcription error, an increase

Fig. 11.4 Microtomy station showing barcode reader and label printer.

in patient safety and a saving of many staff hours in checking and rechecking of numbers. Some of the potential effects upon the workflow resulting from the introduction of this type of system are indicated in (Table 11.2).

Hematoxylin and eosin

Alongside tissue processing, the H&E stain is another major component of histology which is automated in a majority of laboratories. Traditional dip-and-dunk automated H&E stainers come in two basic forms.

The first is the linear style of stainer where the slides progress along a conveyor individually, or within a rack being submerged in various solvent and dye pots. The time in each container is constant, and the stain can be optimized by adapting the number of pots for each solvent and stain (Fig. 11.5).

The streamline design enables a LEAN, first-in-first-out system, and the limited mechanical complexity makes these stainers robust. However, the ability to tailor the staining is limited by the predetermined number of seconds per pot and pot number. Additionally the constant use of the same reagent pots can lead to variation in staining as the day progresses and frequent reagent changes or monitoring may be required. These systems are frequently not fully enclosed, and issues arising from evaporation, humidity and increased user contact with the chemicals can also prove disadvantageous.

The second type is the X-Y stainer design. This is usually enclosed and can attach to ovens and/or a cover slipper. These machines feature a robotic arm which moves the rack along, following a predesigned protocol (Fig. 11.6). Protocols can be designed by dictating the sequence of reagents, and the time in each can be modified independently, allowing multiple stains to be performed on a single unit. User contact with the chemicals can be minimized as the rack can be loaded into an empty input area. For a small number of racks these systems can be quick. One disadvantage of these systems is that unless programs are carefully designed, bottlenecks commonly occur once a certain threshold has been reached. This is because racks are held in a queue for limited heater spaces, or to allow previous racks to complete lengthy staining steps. As a result of this in a larger laboratory these stainers can cause batching of work and delay.

The new generation of automated H&E stainers, such as the Dako Coverstainer and the Roche Symphony, seek to provide a complete integrated system from slide drying to staining and coverslipping. Emphasis is placed upon the reduction of carryover, consistency of staining, LEAN workflow and capacity. Slide racks are typically smaller than those of previous machines, and slides are held flat, side-by-side to reduce carryover and simplify the mechanics of coverslipping. These tend to be 'closed' systems. They require proprietary reagents to be purchased from the manufacturer, but there is the possibility of linking into specimen tracking systems to extend the audit trail.

Table 11.2 The effect of slide transcription automation upon workflow

Stage	Without transcription automation	With transcription automation
Microtomy	Check block. Transcribe block number to slide in pencil. Initial slide. Indicate intended stain on slide. Cut block and mount section on slide. Check transcribed number.	Check and scan block. Cut block and mount section(s) on slide(s) generated.
Post stain labelling	Check handwritten slide number against block number and tissue. Find LIMS generated printed label. Label slide with printed label.	N/A
Post stain quality assurance step	Check label against block. Check slide for section depth and defects. Check slides against request form and compile case. Check slides and form against LIMS system. Release to Pathologists.	Check slide for section depth and defects. Check slides against request form and compile case. Check slides and form against LIMS system. Release to Pathologists.
Audit	Who embedded? – check bead in cassette. Which embedder? – guess work. Who trimmed? – check logbook. Which trimming microtome? – Unknown. Who cut? – check slide. Which microtome? – Unknown. Who checked? – check request form audit trail.	Interrogate database

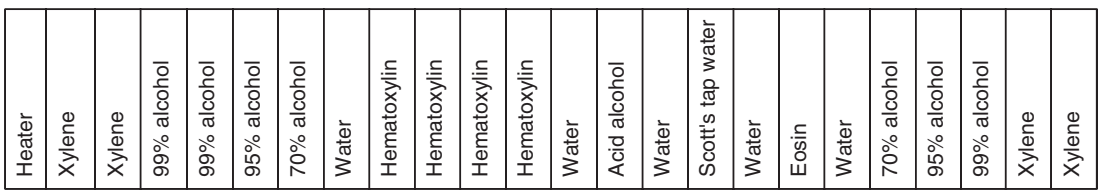

Fig. 11.5 Typical arrangement of reagents in a linear stainer. Slides travel from left to right at a constant speed.

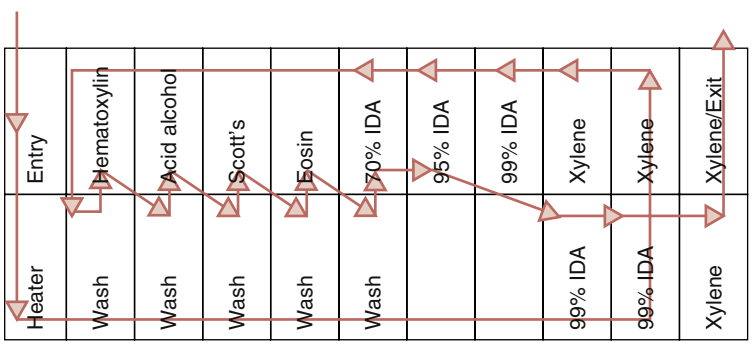

Fig. 11.6 A typical arrangement of reagents in an X-Y stainer. One possible programmed path is shown by the red line.

With so many automated H&E systems available on the market it may be useful to consider the following options when choosing which is right for your laboratory and its workflow:

- Extraction vs. filtration of solvent fumes.
- Speed of single rack vs. speed of 5+ racks.
- Fully autonomous vs. speed of multiple rack.
- 1 stain vs. 2-3 stains in one unit.
- Cost of equipment vs. predicted time savings.
- Compatibility with existing laboratory hardware/software.

Tinctorial staining

Manual staining is a time consuming process which requires at least one trained individual to spend a large proportion of the day juggling timers, stains and rinse steps, often having to adapt the protocol due to tissue type, humidity, dye variation or other variable. If individuals are on a rota through this section, some of the less common stains may not be performed by an individual for months or more, making standardization and competency hard to maintain.

With the increasing need to categorically demonstrate the validity of results, the standardized, same day result which is produced by a tinctorial autostainer can be a great asset to a laboratory. Many of these automated tinctorial staining systems are capable of being customized. Individual slide heaters and the ability to produce a custom protocol enables consistency, whilst also catering to established pathologist preferences.

The overall TAT for individual stains is frequently longer than the bench equivalent (with a few notable exceptions), but the true saving is that of personnel, who are only required to load, unload and maintain the machine. As well as being less labor intensive, these steps do not require the same experience level or length of training as bench staining itself does, reducing the training burden on the laboratory and enabling staff to become competent in the section in a reduced timeframe. As always, a back-up has to be considered in case of technical failure.

There is also the potential for remote requesting: many systems when used together with 3D barcodes and an LIMS system enable the pathologist to request further work from remote workstations. These systems can also resolve some commonly found poor practice, such as the need for illegible paper slips to request further work, stains being missed, or re-labelling of slides by automatically adding requests to both the pending label printing software and pending staining protocols worksheet. Once the barcode labelled slide is added to the stainer it proceeds to confirm and log which stain is required and if the required reagents are both available and in date, information which forms part of the comprehensive audit trail.

Two widely used histochemical stainers are the Dako Artisan Link System (Fig. 11.7) and the Ventana Benchmark SS, both of which use proprietary reagents, individual heating plates and the ability to perform multiple stains in a single run (Roche 2013a; Dako, 2016).

In both cases the emphasis is placed upon the quality and reproducibility of the staining. Reagent management and validation is simplified with the use of barcoded IVD marked reagents. The drawbacks of tinctorial stain automation at present include the limited range of stains available, and the comparatively high cost per slide of automated staining when compared to the conventional manual approach (Table 11.3).

Immunohistochemistry

Immunohistochemistry (IHC) staining (see Chapter 19) revolves around sequential, well-defined steps, time intervals and prescribed temperatures so the whole process is ideal for automation. Indeed, in most laboratories this now happens in one form or another. Many different systems exist which can perform some, or all of the multistep process.

The instruments are a combination delivery system with or without heat, controlled by computer software. Slides may be stationary or mobile and either in a linear or rotary fashion, whilst reagents are delivered via pipettes from reagent containers/pre-packed cartridges. Some platforms also include deparaffinization, multiple antigen retrieval options and counterstain. All of these enable a wide range of immunohistochemical stains to be produced.

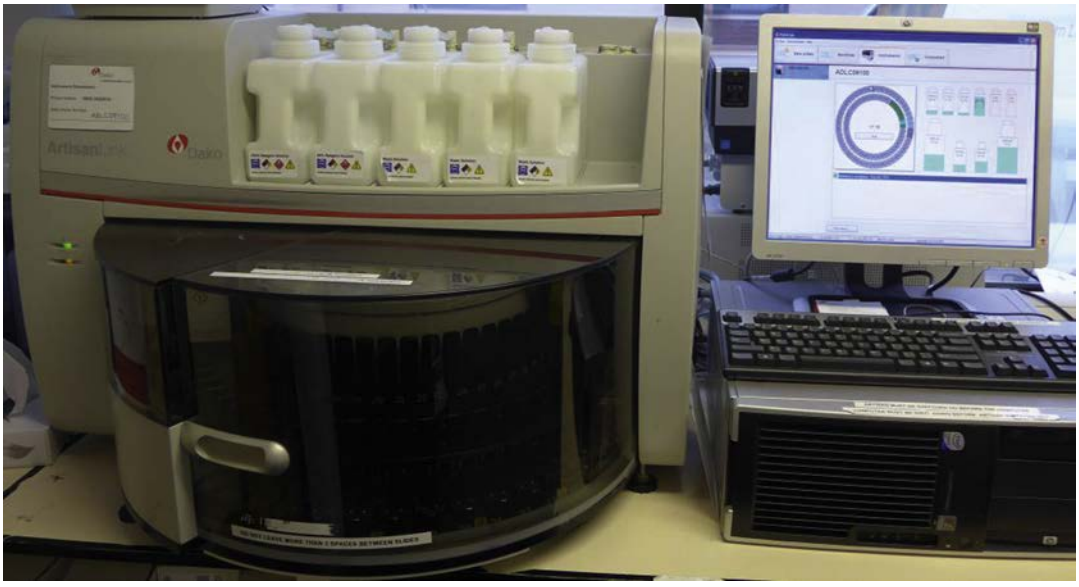

Fig. 11.7 Dako's Artisan Link.

Table 11.3 A comparison of two commercially available automated tinctorial staining platforms to manual staining

	Manual	Bench Mark SS	Artisan
Slide capacity	Limited by space, time and reproducibility	1-20	1-48
Reagent capacity	Limited by space and safety	25	50
Unmanned runs	No	Yes	Requires advance deparaffinization of section
Overnight runs	No	Yes	Yes, but reduces life span of reagents recommended to be refrigerated
Reduces contact with harmful components	No	Yes	Yes

Automation of immunohistochemical techniques has been a major step in improving quality efficiency and reproducibility of results. Most IHC automated systems include flexibility for continuous flow and user defined steps. Some are capable of performing all the steps within the process, making them a true 'walk-away' system requiring far fewer man hours than previous manual or partly automated systems. Workflow features such as independent slide draws and reagent racks are essential if a 'first-in-first-out' workflow system is desired, and work can be continuously added and sent out.

Integration of the platform's software with the LIMS system can allow a full patient tracking package and remote requesting options whilst also removing the need for constant re-labelling of slides. More advanced platforms are available with an expanded range of functions, such as in situ hybridization and direct immunofluorescence.

Examples of IHC platforms on the market include the DAKO Autostainer plus, Labvision Autostainer 720, Ventana Benchmark and Vision Biosystems Bondmax.

Molecular techniques

In-situ hybridization (ISH) and other molecular tests (see Chapter 20) are increasingly used techniques, where specific nucleic acids are targeted within formalin-fixed tissue sections. Individual gain/loss of gene expression can be viewed with both temporal and spatial information, and the targets can either be RNA or DNA.

Many similarities exist in the process of staining to that of IHC. ISH requires timed incubation periods, unique slide reagent management, and rinsing at specified intervals. Additionally a controlled heat application is needed to denature and anneal the nucleic acids, making automation the obvious choice.

Various instruments exist on the market with a range of capacities and abilities and the following are examples. The BioGenex Xmatrix Infinity is a staining system which can provide either IHC, ISH, SS, IF or multiplex staining, as well as slide-based PCR and miRNA. This system fully automates the run from dewax to final coverslip, producing up to 100 slides per day. The Ventana Benchmark ULTRA can provide simultaneous IHC, ISH, SISH, Dual Stain and FITC slide processing, and titration. The latter features individual slide drawers and continuous access to slides, processing up to 90 slides in 8 hours, or 120 slides with an overnight run (BioGenex, 2015; Roche, 2013b).

Slide digitization

Digital images are a common part of daily life, but it is only relatively recently that technology allows the digitalization of an image with sufficient resolution for diagnostic use, and the potential to remove the microscope from the reporting process. This is discussed in more detail in Chapter 22.

In most laboratories tissue slides have to be physically taken to the pathologist, or the pathologist has to travel to view them. This results in potential delays and the added risk of slides being damaged or lost. For a second opinion this process must be repeated with only a single copy of the slide.

Once a slide has been produced and stained, automated slide scanners can scan it and produce high resolution images, as vivid as those seen down a microscope. The hardware and software to perform this is an emerging market, and is becoming available from a growing range of manufacturers, including, for example, Nikon, GE and Hamamatsu.

Currently the storage of the resulting image is a stumbling block to the routine use of digitization. To produce a scanned image, it is necessary to either identify an area of interest in a low-power scan and re-scan these areas at a higher power, or undergo a space-consuming 'whole slide' high-power scan. To compound the issue, most software and hardware can be used to scan at multiple levels, allowing 'z-stacking' of images to duplicate the effect of focusing up and down through the tissue.

GE have released the Omnyx flatbed scanner with a 'load-and-go' operation which enables continuous scanning (overnight if required) to produce images in a timely manner, allowing their potential use for a diagnosis to become realistic for the first time (Fig. 11.8).

Whichever way the images are produced, the ability to view a sample in high resolution on a computer screen removes many of the current limitations:

- Slide images are instantly available anywhere
  - They cannot be left in another office or at another site.
  - They cannot be lost or broken in transit on their way for a second opinion.
  - They do not need to wait for a specialist pathologist to be on site.
- They can be viewed by multiple people at once so they do not miss this week's MTD meeting whilst in transit for the second opinion.
- A patient's records from radiology through to histology can be viewed with ease at MDTs, even those across sites via video conference.
- Pathologists do not need to be on site to report urgent cases.
- Pathologists do not need to wait for an old case to be searched for in an off-site storage facility, they can access it in minutes and do side by side comparisons with the up to date case.

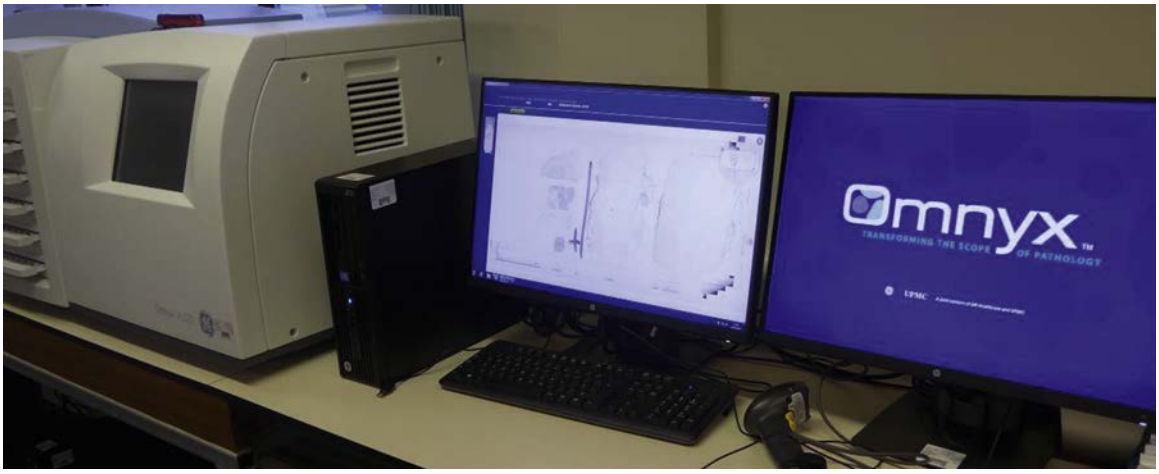

Fig. 11.8 GE's Omnyx flatbed scanner.

Computer algorithms are also under development designed to assist with quantitative and qualitative tasks currently done by eye, such as tumor grading and IHC scoring.

Automated block filing/ archiving

The potential for automation does not stop with the final report. For example, the UK RCPath guidelines recommend that all surgical blocks are kept for up to 30 years. This means that archiving is an expensive and time-consuming problem in many institutions.

There are two approaches to automated archiving of blocks. In the first, the block is scanned and a computer system identifies the next available position for storage. The block is then archived in this position, either using robotic placers or manual placement. The second approach, such as that seen in the Thermo Fisher Arcos system is to mount a scanner above the block file tray, and scan whole racks of barcoded blocks into file. The archive database software then stores the location of each block (Thermo Scientific, 2016).

Similar to other specimen tracking systems, automation of the archival process can be achieved either by development of an in-house IT system or purchased as an off-the-shelf commercial solution. A key feature of these systems is that filing is chronological, NOT numerical. Not only does this reduce the time spent sorting and organizing the archive, it also removes a drawback which has been observed since the onset of electronic requesting systems such as order-comms, where laboratories may be receiving requests already numbered in a non-sequential fashion. With automated archiving there would be no need to relabel these specimens with additional numbers.

The future of automation in histology

What does the future of histology hold? With the current healthcare environment putting cost pressures upon histopathology laboratories to improve efficiency and consolidate resources, the scalability of many processes currently carried out seems likely to be placed under increasing pressure.

Increasing the size of a laboratory and streamlining its workload without also investing in developing the infrastructure underpinning the service, often results in a decline in efficiency and performance. It has been known for some time in many fields that the scalability of a system is often related to the proportion of processes within the system which can be automated. Automation can drive growth, quality, efficiency and standardization of processes whilst increasing the overall capacity of the system (Adler et al., 1995).

Completely integrated systems covering the histology process from receipt to reporting are already on the way, and slide scanning is developing

towards a point where it will soon be conceivable to use and store digital images in place of glass slides. Techniques and software such as telepathology, voice recognition and macroscopic imaging can all be combined and made available to the reporting pathologist alongside the digital slide to produce an integrated reporting platform which can be remotely accessed by multiple users simultaneously.

The laboratory process itself can be streamlined, with sample tracking, automated transcription and remote test requesting increasing patient safety, whilst reducing the workload.

Reproducibility, consistency and reagent management can be improved and recorded through use of automated processing and staining platforms. These techniques can produce audit data which can be used to identify bottlenecks, target inefficiencies and monitor workflows visible in real time on 'dashboard' style displays.

All this is currently theoretically possible and elements have been in use in some laboratories for over a decade. However, the uptake of these technologies has been slow in many laboratories and must not be seen as a quick fix.

The automated instrumentation and workflow patterns must work synergistically together and address the following:

- Will a stainer with attached heater stations release a scientist from the need to load slides onto a stainer or delay slides if the workload is greater than the heater's capacity?
- Will the limited repertoire of tinctorial stains provided by an automated system streamline the process, or result in the need to run two systems in parallel?
- Will being tied into a closed system where reagents can only be purchased from one supplier result in increased cost, or does the known standardization and release of staff from making these reagents lead to an overall saving?

If a task can be done manually more consistently, faster and at reduced cost to an automated system then automation in that instance is often not the preferred outcome.

It is also worth considering that laboratory automation is rarely a low cost initiative. As a result, it is often introduced in a fragmentary fashion as funding becomes available and equipment requires replacement. This piecemeal introduction can be particularly inefficient and frustrating when it is considered that the largest benefits of automation are realized when each element is linked together to form a cohesive system.

It is the role of the laboratory management team to bear in mind the holistic vision and overall objective of the department when considering such replacements to ensure unified and efficient adoption of improvements in technology. Each component must be selected to both add value in itself, but also represent a step towards a long-term goal of an integrated system. Automation as a service development is dauntingly expensive in terms of resource commitment, requiring large amounts of funding and/or time to be invested in the process. Despite this the ever-increasing potential for the realization of improvements in efficiency, economy, quality and safety which is offered by the intelligent use of automation in histology makes it a significant option for a modern laboratory.

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Connective and other mesenchymal tissues with their stains

12

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Introduction

During embryonic development, the ectoderm and endoderm are divided by a germ cell layer, the mesoderm or mesenchyme. The term mesenchyme comes from the Greek 'mesos' meaning middle and 'enchyma' meaning infusion. Connective tissues and muscle develop from the mesenchyme. The parent cell of the entire series, the embryonic mesenchyme cell, is rarely found in adults.

Connective tissue

This is one of the four tissue types found in the body, the term coming from the Latin 'connectere' meaning to bind. Its main function is to connect together and provide support to the other tissues. Most types of connective tissue consist of three elements: the cells, the fibers and the amorphous ground substance which the cells produce. The histological identification of the cells is based upon their appearance in areolar and loose tissues, the main 'packing' material in the adult.

Connective tissues are divided into the following groups:

- Connective tissue proper
  - Loose: areolar, adipose (brown or multilocular and white or unilocular) and reticular.
  - Dense: regular, e.g. ligaments, tendons and joint capsules and irregular, e.g. dermis.
- Cartilage – hyaline, elastic and fibrocartilage.
- Bone – spongy (cancellous) and dense (cortical) (See Chapter 17).
- Blood.
- Blood-forming – hematopoietic (NB blood and hematopoietic tissues are not discussed in this book as it is a large, complex and specialized area).

Connective tissue usually consists of a cellular portion in a surrounding framework of a non-cellular substance. The ratio of cells to intercellular substance and the primary function varies from one type of connective tissue to another, e.g. bone has few cells in a usually dense, rigid intercellular substance, its main function being strength and support. The cell types of connective tissue include fibroblasts, mast cells, histiocytes, adipose cells, reticular cells, osteoblasts, osteocytes, chondroblasts, chondrocytes, blood cells and blood-forming cells. Many of these can be analyzed using immunocytochemical techniques (see Chapter 19).

From the histochemistry perspective, connective tissues are usually considered as intercellular substance. This is usually composed of both amorphous, non-sulfated and sulfated mucopolysaccharides, and the formed elements of collagen, reticular fibers and elastic fibers. These are the non-cellular parts of the connective tissues. The nature of the intercellular substance varies according to its function. It may be extremely hard and dense as in cortical bone or soft as in the umbilical cord. Its microscopic appearance also varies from fibrillar to homogeneous allowing its classification into two main groups:

- Formed or fibrous types
- Amorphous or gel types.

Formed or fibrous intercellular substances

A frequent fault among histologists is to speak of collagen, reticulin and elastin, when in reality they mean collagen fibers, reticular fibers and elastic fibers. The former terms relate to the protein compound which is predominant in the particular fiber and should not be used to describe the connective tissue fiber itself.

Collagen fibers

These are the most frequently encountered of all the fibrous types of intercellular substance. They can occur as individual fibers, e.g. in loose areolar tissues where they are arranged in an open weave system, or as large bundles of fibers clumped together to form structures of great tensile strength, e.g. in tendon. Individual collagen fibers do not branch and when viewed by polarized light they are strongly birefringent, but lack dichroism.

Types of collagen

There are four major types of collagen (designated *I–IV*), although several minor types are recognized. The production of the different types is under genetic control, each reflecting slight variations in the α-chain composition but all displaying the same characteristic amino acid content.

Type I

This forms the thick collagenous fibers which have been demonstrated histologically and form the bulk of the body's collagen. It appears under the electron microscope as bundles of tightly packed thick fibrils, 75 nm diameter, with little interfibrillar substance. The fibrils show the characteristic 64 nm axial periodicity. The prominence of the 'cross-banding' in Type I collagen is thought to be due to the lack of interference from interfibrillar ground substance (Fig. 12.1).

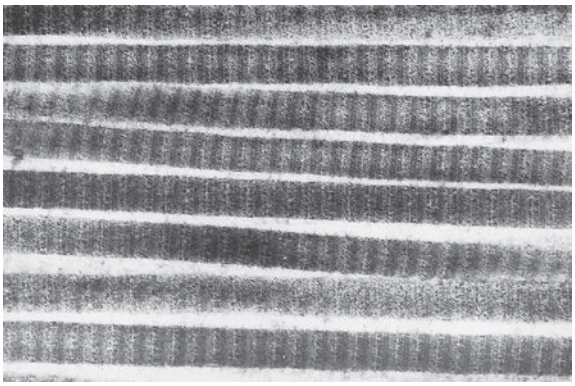

Fig. 12.1 Electron micrograph of human collagen showing transverse cross-banding.

Type II

This is found in hyaline and elastic cartilage and is produced by chondroblasts. The fibers are thin and composed of fibrils arranged in a meshwork with copious amounts of proteoglycans. It is usually not visible by light microscopy. The Type II fibers found in articular cartilage are thicker and ultrastructurally resemble Type I fibers. The cross-banding of Type II collagen is less evident due to the masking effect of the abundant interfibrillar material.

Type III

This collagen, known as reticular fibers, is found only in those tissues which also contain type I collagen, e.g. lung, liver, spleen and kidney. Ultrastructurally, reticular fibers are loosely packed fibrils surrounded by abundant carbohydrate-rich interfibrillar material. The argyrophilia of reticular fibers is due to the proteoglycan content of the fibers and is not dependent upon the proteins of the fibrils themselves. Type III collagen provides a limited amount of support, but allows some motility and the easy diffusion and exchange of metabolites.

Type IV

This has been characterized in structures identified morphologically as basement membranes. It is generally accepted that it does not form fibers or fibrils visible with light microscopy. Electron microscopy reveals a random organization of fine fibrils forming a feltwork-like structure in all basement membranes. Type IV collagen is associated with large amounts of carbohydrate complexes explaining the strong reaction of basement membranes to the periodic acid-Schiff (PAS) method.

Types V and VI

These are similar. Type V collagen is produced in small quantities by a wide range of cells which include connective tissue, endothelial and some epithelial cells. It remains in close contact with the cell surface and is thought to be involved in the attachment of cells to adjacent structures and the maintenance of tissue integrity. Type VI collagen is a disulfide-rich variant which has been identified in boundary zones where

interstitial collagenous fibers (types I and II) are linked to non-collagenous elements.

Type VII
This is the major structural component of anchoring fibrils which secure the basement membrane to the underlying dermis.

Staining reactions of collagen

Type I collagen stains strongly with acid dyes due to the affinity of the cationic groups of the proteins for the anionic reactive groups of the dye. Collagen may be demonstrated more selectively by either compound solutions of acid dyes, e.g. van Gieson, or by sequential combinations, e.g. Masson's trichrome and Lendrum's MSB. The different types of collagen may be differentiated immunohistochemically.

Reticular fibers

These are the fine delicate fibers which are found connected to the coarser and stronger collagenous type I fibers. They provide the bulk of the supporting framework of the more cellular organs, e.g. spleen, liver and lymph nodes, where they are arranged in a three dimensional mesh network providing a system of individual cell support (Fig. 12.2). On light microscopic examination, reticular fibers are weakly birefringent, the weak reaction being attributed to their lack of physical size and the masking effect of the interfibrillar substance. They are seen to branch frequently but appear indistinct in H&E stained preparations. Reticular fibers may be demonstrated in paraffin sections using an argyrophil-type silver impregnation technique, or in frozen sections by the PAS technique. Both methods of demonstration are dependent upon the reactive groups present in the carbohydrate matrix and not upon the fibrillar elements of the fiber.

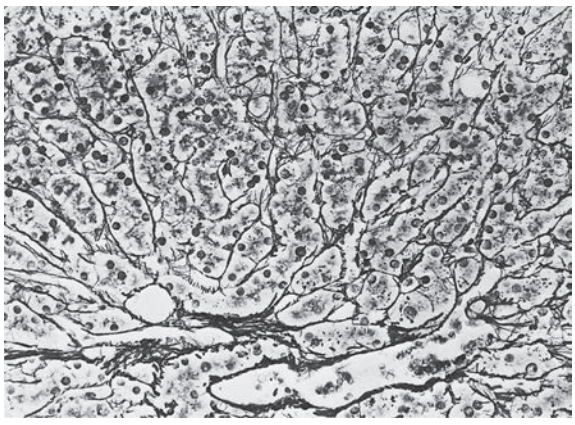

Fig. 12.2 Reticular fiber pattern in normal liver by Gordon and Sweets' (1936) silver impregnation method.

Elastic fibers

The elastic system fibers oxytalan, elaunin and elastic have a fibrillar, amorphous, or mixed structure respectively. They may be found throughout the body but are especially associated with the respiratory, circulatory and integumentary systems. Their appearances under the light microscope may vary considerably according to their location from fine, single fibers as in the upper dermis to the membrane-like structures of the internal and external elastic laminae in the large arteries. In the latter, the elastic membranes are interrupted by minute holes called fenestrae (from the Latin '*fenestra*' for window). These fenestrae permit diffusion of materials through the otherwise impermeable membrane. High resolution electron microscopic examination has demonstrated that elastic fibers consist of two quite distinct components: the amorphous substance which biochemically is consistent with the protein elastin, and a second component which shows a periodicity of 4–13 nm and is microfibrillar in nature, the elastic fiber microfibrillar protein (EFMP).

Viewed in transverse section, the central core of the elastic fiber is composed of the amorphous protein elastin surrounded by a ring or band of elastin-associated microfibrils (EAMF). The proportions of the two components alter with the age of the fiber and probably the age of the subject. The dominant fraction in young fibers is the microfibrillar protein but in older fibers, the amorphous protein accounts for over 90%. The basic molecular unit of elastin is a linear polypeptide with a molecular weight of approximately 72 kilodaltons (kDa), 'soluble or tropoelastin'. One of the characteristic features of elastic fibers is the presence of cross-linking which binds

the polypeptide chains into a fiber network. The polypeptides are transported out of the fibroblasts or smooth muscle cells and the cross-linking occurs in the extracellular spaces.

EFMP has an amino acid content which is quite distinct biochemically from elastin protein. It is particularly rich in amino acids which are lacking, or only present in small quantities in elastin. The content of cysteine in EFMP is high because of the presence of numerous disulfide linkages and this is significant for the staining properties of elastic fibers. A number of carbohydrate complexes, 'structural glycoproteins' (Cleary & Gibson, 1983) are also associated with EFMP and significant in the staining of elastic fibers. Elastic fibers are acidophilic, congophilic and refractile. Following oxidation, they are strongly basophilic due to the formation of sulfonic acid groups from the disulfide linkages of the EFMP. Young fibers with a high content of EFMP show a positive PAS reaction. They may be seen in routine H&E stained sections or by the use of a relatively simple stain e.g. the Taenzer-Unna orcein method, or the more lengthy and complex, e.g. Weigert resorcin-fuchsin method. Physical and biochemical changes are seen with increasing age in elastic fibers. These may include splitting and fragmentation, alteration of the ratio of EFMP to elastin and increases in the levels of calcium and glutamic and aspartic acids. These changes are readily visible in skin which becomes wrinkled and 'loose-fitting'.

Oxytalan fibers

These were first described by Fullmer and Lillie (1958) in periodontal membranes, but more recently they have been demonstrated in a wide variety of both normal and abnormal tissues (Alexander & Garner, 1977; Cleary & Gibson, 1983; Goldfischer et al., 1983). Unless they have previously been oxidized by potassium permanganate, performic acid or peracetic acid, oxytalan fibers may be distinguished from mature elastic fibers by their failure to stain with aldehyde fuchsin solutions on light microscopy. They have also been reported to remain unstained following Verhöeff's hematoxylin, with or without prior oxidation. Under electron microscopic examination oxytalan fibers appear similar, if not identical to, EFMP fibers. From their morphology, localization, and staining properties, it is possible that oxytalan fibers represent an immature form of elastic tissue. Goldfischer et al. (1983) suggested that microfibrils and oxytalan fibers may have a role beyond that of elastogenesis which involves 'anchoring' mechanisms between e.g. collagen fibers, stromal cells, lymphatic capillary walls, mature elastic fibers and muscle cells.

Elaunin fibers

Gawlik (1965) first described these fibers which, unlike oxytalan fibers, stain with orcein, aldehyde fuchsin, and resorcin–fuchsin without prior oxidation, but do not stain with Verhöeff's hematoxylin.

It is suggested that the differentiation by staining between elaunin and oxytalan fibers is too empirical and that there is lack of structural or functional difference between them. The three fiber types, oxytalan, elaunin, and elastic may correspond to consecutive stages of normal elastogenesis. It has been shown that there is continuity between the coarse, mature elastic fibers deep in the dermis through the intermediate elaunin fibers, to the fine oxytalan fibers in the superficial aspects of the papillary dermis.

Basement membranes

These are found throughout the body as a resilient matrix separating connective tissues from epithelial, endothelial, mesothelial, muscle or fat cells and nervous tissues. They support the epithelial cells of mucosal surfaces, glands and several other structures and also the endothelial cells lining blood vessels. The basement membrane is not homogeneous but divided into three zones or layers:

- **Lamina rara or lamina lucida:** adjacent to the surface cells and composed mainly of carbohydrate complexes. It is apparently continuous with the glycocalyx of the surface cells and thought to be

produced by the surface cells and not by the underlying connective tissue cells.

- **Lamina densa or basal lamina:** composed of a feltwork of microfibrils which have been immunohistochemically identified as predominantly type IV collagen with a lesser amount of type V collagen. Type IV collagen is associated with relatively large amounts of structural glycoproteins, mainly laminin and fibronectin, and small amounts of proteoglycans, principally heparan sulfate (Junqueira & Montes, 1983; Laurie & Leblond, 1983).
- **Lamina reticularis:** a layer containing fibrous elements which are continuous with the underlying connective tissue fibers.

The thickness of the basement membrane varies from site to site but most are 15–50 nm thick. The kidney glomerular basement membrane (GBM) however is particularly thick, averaging 350 nm in a healthy adult. The ultrastructural appearance of the GBM also differs from that of other basement membranes, the central lamina densa is bordered on both sides by a lamina rara. These membranes are strongly positive with the PAS reaction and any other oxidation-aldehyde demonstration techniques, e.g. methenamine silver, Gridley and Bauer-Feulgen, as a result of their carbohydrate content. The basement membrane can also be stained by MSB or Azan trichrome methods.

Methenamine silver microwave method

Methenamine silver demonstrates the carbohydrate component of glomerular basement membranes by oxidizing the carbohydrates to aldehydes. Silver ions from the methenamine-silver complex are first bound to carbohydrate components of the basement membrane and then reduced to visible metallic silver by the aldehyde groups. Toning is achieved with gold chloride and any unreduced silver is removed by sodium thiosulfate. The use of a microwave oven is recommended for the method and the technique should be followed exactly for optimal results. The method below is for five slides. If you do not have five slides include blank slides but do not use more than five.

Periodic acid-methenamine silver microwave method for basement membranes (Brinn, 1983; Carson, 1997)

Fixative

10% neutral buffered formalin is preferred. Mercury-containing fixatives are not recommended.

Sections

Paraffin-processed tissue cut at 2 µm.

Solutions

Stock methenamine silver

3% aqueous methenamine	400 ml
5% aqueous silver nitrate	20 ml

Keep refrigerated at 4°C.

5% borax (sodium borate) solution

Working methenamine silver solution

Stock methenamine silver	25 ml
Distilled water	25 ml
5% borax	2 ml

1% periodic acid solution

0.2% gold chloride solution

1% gold chloride	1 ml
Distilled water	49 ml

Stock light green solution

Light green SF (yellowish)	1 g
Distilled water	500 ml
Glacial acetic acid	1 ml

Working light green solution

Light green stock solution	10 ml
Distilled water	50 ml

Method

1. Deparaffinize sections and rehydrate to distilled water.
2. Place sections in 1% periodic acid solution for 15 minutes at room temperature.
3. Rinse in distilled water.
4. Place 5 slides in a plastic Coplin jar containing 50 ml of methenamine working solution. Loosely apply the screw cap and place in the microwave oven, and place a loosely capped plastic Coplin jar containing exactly 50 ml of distilled water in the oven. Microwave (1000 Watt) on full power for exactly 70 seconds (see Note b). Remove both jars from the oven, mix the solution with a plastic Pasteur pipette and let stand. Check the slides frequently until the desired staining intensity is

achieved. This will take approximately 15–20 minutes in a 1000 Watt microwave but time calibration may be required (see Note b).
5. Rinse slides in the heated distilled water.
6. Tone sections in 0.02% gold chloride for 30 seconds.
7. Rinse slides in distilled water.
8. Treat sections with 2% sodium thiosulfate for 1 minute.
9. Wash in tap water.
10. Counterstain in the working light green solution for $1^1/_2$ minutes.
11. Dehydrate with two changes each of 95% and absolute alcohol.
12. Clear with xylene and mount with synthetic resin.

Results

Basement membrane	black
Background	green

If a microwave oven is not used, substitute the following solutions and staining times:

Methenamine silver solution

Stock methenamine silver solution	50 ml
5% borax	5 ml

Preheat the solution and stain slides at 56–60°C for 40–90 minutes.

0.2% gold chloride solution

Gold chloride, 1% solution	10 ml
Distilled water	40 ml

Notes

a. Sharper staining of the basement membrane and less background staining can be obtained with the use of the microwave oven for silver techniques.
b. The temperature is critical and should be just below boiling, approximately 95°C, immediately after removal from the oven. Each microwave oven should be calibrated for the time required to reach the correct temperature.
c. This is a difficult stain to perform correctly. The glomerular basement membrane should appear as a continuous black line. Stopping the silver impregnation too soon will result in uneven or interrupted staining. The application of too much counterstain will mask the silver stain and decrease contrast.

Connective tissue cells

As previously stated, connective tissues consist of two elements, the framework and the constituent cells. The parent, or progenitor cell of the entire series of connective tissues is the undifferentiated mesenchymal cell. This produces a range of connective tissue cells, each with their own different function.

Fibroblasts

This cell is responsible for the production of collagen fibers and probably the amorphous intercellular substance which binds the fibers together. Many authors refer to the young active secretory cell as the fibroblast, and reserve the term fibrocyte for the older non-secretory stage of development. The two stages may be distinguished under the microscope. The nucleus of the active spindle shaped fibroblast contains a prominent nucleolus and is surrounded by abundant, slightly basophilic cytoplasm. The thinner spindle shaped fibrocyte has an ovoid flattened nucleus with scanty chromatin, no nucleolus and difficult to distinguish cytoplasm. The fibroblasts are responsible for repair processes in the body and will accumulate at the edge of sites of injury and secrete fibrous intercellular substances which ultimately form scar tissue.

Fat cells or adipocytes

These are unique in the cells which differentiate from the mesenchymal cell as their main function is storage and not the production of intercellular substances or defense mechanisms. The first sign of development of a fat cell is the accumulation within its cytoplasm of tiny droplets of lipid material. These gradually increase in size until the cell loses its previous shape and appears as a swollen object with the nucleus and residual cytoplasm forced to one side.

Areolar tissue

This is the most widespread of all the connective tissue types and connects the epithelial surfaces to the underlying structures. It also fills spaces between organs and forms the fascia of intermuscular planes. It has considerable strength but its structure allows

movement of adjacent structures relative to each other. The loose pattern of areolar tissue permits free passage of nutrients and waste products. In a stained section under the microscope, areolar tissue appears as an open-weave network of numerous single or small bundles of collagen fibers running in all directions, with some elastic and reticular fibers. The most frequent cells are the fibroblasts which lie adjacent to a fiber or bundle with small numbers of mast cells and macrophages. There are numerous arterioles, blood and lymphatic vessels.

Adipose tissue

This is a loose connective tissue which is not directly concerned with support or defense functions. It evolves from areolar tissue as adipocytes replacing almost all of the other cells and many of the fibers. There is a well-developed network of collagen reticular fibers surrounding the fat cells, elastic fibers are almost absent. Adipose tissue is well supplied with capillaries and lymphatics as it is associated with the storage of excess nutrients. Microscopically, it is different to other body tissues as it appears as a collection of cells with flattened eccentric nuclei and in paraffin wax preparations, clear spaces where the lipid has been removed during processing.

'Myxoid' connective tissue

This is one of the less commonly encountered connective tissues and is not normally found in adult humans. It is found in the umbilical cord as 'Wharton's jelly' and as part of a variety of disease states including some neoplasms, e.g. cardiac myxoma and liposarcomas. It is a cellular tissue with stellate fibroblasts which anastomose and are embedded in a mucinous intercellular matrix containing hyaluronic acid. There are few collagen fibers apart from those in the blood vessels.

Dense connective tissue

This is seen as the capsules enclosing organs and, in particular, tubular structures, but is most strikingly characterized in its appearance as tendons and ligaments. These are basically dense masses of collagen fibers and fibroblasts arranged in an orderly manner, with the cells and fibers being oriented in the same direction parallel to the long axis of the tendon. Originally there is a predominance of fibroblasts, but these secrete increasing amounts of collagen and the bulk of the tendon becomes fibrous. These structures have enormous tensile strength and are perfectly suited for connecting the skeletal muscles to the skeleton and transmitting power.

Cartilage

This develops from the mesenchymal cells which differentiate into chondroblasts and lay down intercellular substance. Chondroblasts mature into chondrocytes and the cartilage formed can live for long periods, e.g. in joints.

The connective tissues discussed previously possess great tensile strength but, when placed under pressure, they will bend. The structural characteristics of cartilage partially overcomes this problem. It consists of a dense network of collagen fibers encased in, or bonded with, an amorphous intercellular substance of chondroitin sulfate which is in the form of a thin gel. Cartilage is distributed throughout the body in sites where it performs slightly different functions. It has a 'standard' form, hyaline cartilage, and two other modified forms, elastocartilage and fibrocartilage.

Microscopically hyaline cartilage is composed of a matrix of homogeneous intercellular substance, fibrillar in structure and containing large numbers of collagen fibers. The cellular components of cartilage, the chondrocytes, reside in spaces in the matrix known as 'lacunae'. There may be one cell or as many as six cells in each lacuna. In a fresh state the chondrocytes will fill the lacunae but in stained sections they will appear shrunken. The cytoplasm contains glycogen and lipids; the nuclei are spherical with one or more nucleoli.

Hyaline cartilage is the most common cartilage, it is found in the larynx, bronchi, nose and the articular surface of joints. When thoroughly lubricated with synovial fluid, the surface of articular cartilage appears highly polished and is ideally suited for the opposing surfaces of joints.

Hyaline cartilage is slightly elastic but elastocartilage is found where more elasticity is required. It

contains as many collagen fibers but has the addition of elastic fibers, e.g. in the external ear and epiglottis.

Fibrocartilage is found, e.g. at tendon insertions, where the tensile strength of hyaline cartilage is insufficient. The collagen fibers of hyaline cartilage are arranged with no regular pattern but in fibrocartilage they are packed in rows parallel to the direction of the force. Fibroblasts and rows of chondrocytes lie within the intercellular substance between the collagen fiber bundles.

Bone

Cartilage in its several forms is capable of providing support and resisting converging forces. Calcified cartilage is much stronger, but as the process of calcification occurs the chondrocytes are cut off from their nutrients which come through the permeable intercellular structure, and they die. A permanent rigid type of connective tissue, bone is required to support the body's weight, to maintain its optimal shape and to shield its delicate structures from external damage. The structure of bone is discussed in detail in Chapter 17.

Other mesenchymal tissues

Muscular tissue

This provides the power to enable the body to move and to function. Muscular tissue is divided into three distinct categories but all types are composed of similar constituents and their mode of providing power and movement is also similar. Muscles provide power and movement by contracting their cells, shortening their overall length, and thus pulling the points where the muscle is attached closer together. Many cells in the body share this ability to contract and change shape and this is due to the presence of three proteins and the interaction between them:

- α-Actin
- Actin
- Myosin.

α-Actin forms a base which allows a strand of actin to become attached. Myosin in turn attaches to this actin strand and is able to move along the strand towards the base by means of a ratchet like mechanism and, as it is double headed, it interacts simultaneously with two separate actin strands. The movement up the strands pulls the strands together, causing the fiber to contract. A single fiber of skeletal muscle is long and thin comprising of many myofibrils which run parallel to its long axis. Each myofibril is built up of a large number of identical contractile units called sarcomeres and, on full contraction these can shorten its resting length by approximately 30%.

Muscular tissues are classified into three types:

- Involuntary smooth muscle
- Voluntary striated muscle
- Striated cardiac muscle.

Involuntary smooth muscle

This develops from mesenchymal cells which elongate themselves to form tapered cells of 20–50 μm in length. It is found in the bowel, skin and bladder where it is involved in automatic peristalsis and reflex functions. The size of the muscle cell, or fiber, varies enormously depending upon the site where the cells are found. The cytoplasm contains bundles of myofilaments called myofibrils which are up to 1 mm in diameter. These are surrounded by the sarcoplasm, which constitutes the remainder of the cell. The centrally situated nuclei with their fine chromatin pattern are lightly stained with hematoxylin and have an elongated appearance which may contain one or two nucleoli. In cells which contract extensively and regularly, the nuclei may take on a 'concertina' shape which enables them to fit comfortably into the cell when it is in a state of full contraction.

Voluntary striated muscle

This makes up the bulk of the body's musculature and is widely distributed over all parts of the skeleton, hence its alternative name of skeletal muscle. It is the type of muscle over which there is voluntary control. It is capable of briefly producing tremendous forces and also of maintaining a state of 'semi-contraction' for long periods of time, e.g. holding the body upright whilst standing. Certain functions, e.g. respiration, become almost automatic and driven by

the nervous system although voluntary control is maintained. Like smooth muscle, it is composed of elongated eosinophilic cells, but in striated muscle these are much larger and longer, up to 40 mm in length and 40 µm in diameter. The cells themselves do not taper towards the ends but are more cylindrical. The nuclei are elongated and stain with hematoxylin. There is often more than one nucleus present in each cell and these are situated peripherally and often in contact with the sarcolemma or cell membrane.

The most obvious and striking characteristic of striated muscle is the striations or stripes which cross the cells at right angles to their long axes. On closer examination, and by polarized light and electron microscopy, the striations can be seen to be alternating light and dark bands or discs. The darker bands are birefringent and referred to as A-bands or Q-bands, the paler bands are isotropic and known as I-bands. Occasionally there is a narrow, dark band, the Z-line, running through the center of the paler I-bands. Studies of relaxed voluntary muscle with the electron microscope have shown the presence of a fourth extremely thin band, the H-line or H-disc, running through the A-band (Fig. 12.3). With the aid of the electron microscope it may be noted that these numerous discs or bands are not complete structures crossing the muscle cells, but are composed of striated myofibrils. The organized arrangement of these striae gives the appearance of discs. There is a well-developed system of mitochondria and the sarcoplasmic reticulum between these myofibrils. Stored in the sarcoplasm, adjacent to the sarcoplasmic reticulum, is an abundant store of glycogen to provide an immediate source of energy.

Striated cardiac muscle

This is only found in the heart where it constitutes the bulk of the myocardium and differs from smooth and striated muscle in several ways. It is not composed of distinctly separate cells but of cells which branch and anastomose frequently. The cytoplasm contains myofibrils and sarcoplasm and exhibits a 'striated' appearance similar to that of voluntary muscle. The striations are less distinct as the myofibrils are not arranged to coincide as regularly as in voluntary striated muscle.

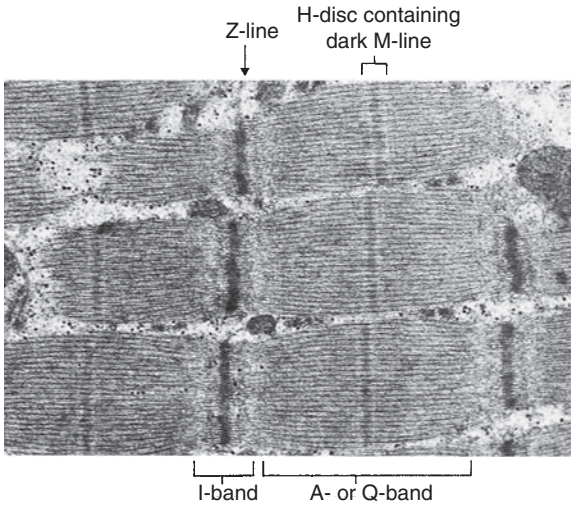

Fig. 12.3 Electron micrograph of human striated muscle showing characteristic pattern of striations.

One feature unique to cardiac muscle is the presence of intercalated discs. These were originally believed to be another type of striation or stripe but with the electron microscope it can be seen that these discs represent the end to end junction of adjacent cardiac muscle cells. The nuclei are placed centrally in the cells and stain lightly with hematoxylin. Situated in the intercellular spaces created between the anastomosing of the cells are the blood vessels, lymphatics and nerves to maintain and nourish the cardiac muscle.

General structure of muscle

All the different types of muscle contain considerable amounts of connective tissue. Each complete muscle is surrounded by an envelope of collagen and elastic fibers known as the 'epimysium'. There are numerous bands or sheets of connective tissue arising from the epimysium which divide the whole muscle into bundles of muscle cells, the 'perimysium'. A connective tissue sheath, the 'endomysium' covers each individual muscle cell. This complex system of interconnecting collagen fibers is continuous with the attachment points of the muscle, the fine sheets of collagen fibers give way to broader, stronger bands of dense connective tissue which continue to form the tendon.

Fibrin and fibrinoid

These are not tissues but the stains used for the demonstration of connective tissues are also used for their identification.

Fibrin is an insoluble fibrillar protein formed by polymerization of the smaller soluble fibrillar protein fibrinogen, one of the plasma proteins. Fibrin is most commonly seen in tissues where there has been tissue damage, e.g. in an acute inflammatory reaction where there is transudation of fluid and plasma proteins out of damaged vessels. The plasma fibrinogen polymerizes at the end point of the clotting cascade to form insoluble fibrin outside the vessels. Fibrin is an important constituent of the acute inflammatory exudate and may be found wherever there is recent tissue damage.

In paraffin sections fibrin is strongly eosinophilic and stains blue-black with Mallory's phosphotungstic acid hematoxylin (PTAH). Lendrum et al. (1962) devised a trichrome method, the MSB, to demonstrate fibrin and to attempt to distinguish between fibrin of varying ages. 'Fibrinoid' is an eosinophilic material which has identical staining reactions to fibrin but occurs in tissues in different situations and disorders. It is frequently found within vessel walls which have undergone acute damage, e.g. 'necrotizing vasculitis'.

Connective tissue stains (Table 12.1)

Trichrome stains

This is a general name for a number of techniques for the selective demonstration of muscle, collagen fibers, fibrin and erythrocytes (Fig. 12.4). Three dyes are employed, one of which may be a nuclear stain. The original methods were used to differentiate between collagen and muscle fibers, and some are still used, van Gieson being one of the earlier techniques still in constant use.

Factors affecting trichrome staining

Tissue permeability and dye molecular size

Little work has been published on the permeability of tissues and the 'pore' size of fixed tissues, but deductions can be made from the reactions of various tissue elements with a range of anionic dyes of differing molecular size (Table 12.2). When the

Table 12.1 Connective tissue stains and their reactions

Tissue	van Gieson	Masson trichrome	MSB	PTAH	PAS	Retic	Meth. silver	Auto fluor.	Refrac.	Biref.	H&E
Muscle	Yellow	Red	Red	Blue	+	Gray	Gray	–	–	–	Deep pink
Collagen	Red	Blue/green	Blue	Orange/ red	+	Gray	–	–	–	+	Deep pink
Elastin	Yellow	–	Blue	Orange/ brown	–	–	–	+	+	–	Pink
Reticulin	Yellow	Blue/green	Blue	Orange/ brown	++	Black	–	–	–	–	–
Basement membranes	Yellow	Blue/green	Blue	Orange	+++	Gray	Black	–	–	–	Pink
Osteoid	Red	Blue/green	Blue	Orange/ red	+	Gray	–	–	–	+	Deep pink
Cartilage	Varies	Varies	Varies	Varies	++	Varies	Varies	–	–	–	Purple
Fibrin	Yellow	Red	Red	Blue	+/–	Gray	–	–	–	–	Pink

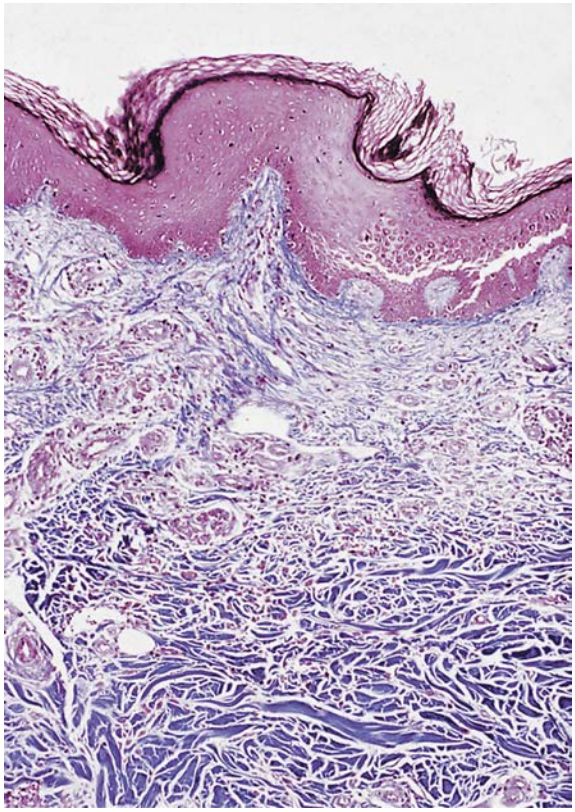

Fig. 12.4 Masson trichrome on a section of skin demonstrating collagen (stained blue) in the dermis.

protein component of a tissue is exposed to a fixative agent, an interaction between the protein chains and the fixative occurs. The nature of the reaction and the end result will vary according to the exact composition of the protein and the fixative. As a general rule, a three-dimensional, insoluble protein 'network' is formed and the different proteins will form networks with different physical features.

The structure and density of the protein network may relate directly to the staining reactions of the tissue components. The smaller molecule dyes will penetrate and stain any of the tissue types. Medium sized dye molecules will penetrate muscle and collagen, but will not react with erythrocytes. The larger dye molecules will penetrate only collagen, leaving muscle and erythrocytes unstained. In practice, the rules seem less rigid, e.g. acid will stain collagen when used in combination with picric acid in van Gieson's stain, but when used with light green SF in Masson's trichrome, it stains erythrocytes and muscle. Some of the large molecular size dyes, e.g. sun yellow and pontamine sky blue 6BX, fail to stain erythrocytes even when the tissues are exposed to the dye solution for long periods. This is due to the incompatibility between the dye molecule size and the permeability of the red cell protein.

The general rule in trichrome staining is that a small dye molecule will penetrate and stain a tissue element but when a larger dye molecule can penetrate the same element, the smaller molecule will be replaced by it. Detailed information regarding the molecular size of dyes is not readily available but the molecular weight may be used as an indication of the relative sizes. It is also possible to calculate the ionic weight of a dye from the molecular weight and this is also of value in estimating relative sizes. Horobin (1980) presents a number of interesting hypotheses relating to tissue structure and its influence on staining mechanisms (see Chapter 9).

Heat

Heat has been shown to increase the rate at which trichrome staining occurs and also to influence penetration by larger dye molecules.

pH

Dyes utilized in trichrome methods are prepared as low pH solutions, often pH 1.5–3.0 to achieve adequate and even staining of connective tissue fibers.

Nuclear stains

Due to the acidity of dye solutions used in the differential staining of connective tissue fibers, standard alum hematoxylins are decolorized in the subsequent treatment. Iron hematoxylins are more resistant to acid solutions and are therefore used in most of the techniques.

Effects of fixation

The use of routine, formaldehyde-fixed tissues for trichrome techniques will not produce optimal results and the results are worse with prolonged fixation. This is due to the tissue groups becoming saturated with formaldehyde leaving few groups

Table 12.2 Dyes in connective tissue stains

Dye	Alternate name	Color index number	Dye formula weight
Picric acid	Trinitrophenol	10305	229
Martius yellow	Acid yellow 24	10315	251
Lissamine fast yellow	Xylene yellow G	18965	551
Sun yellow	Direct yellow 11	4000	837
Orange G	Acid orange 10, Lissamine orange 2G	16230	452
Fast green FCF	Food green 3	42053	809
Fast light green	Cyanol green B, Acid green 50	44090	577
Light green SF	Acid green 5	42095	793
Acid fuchsin	Acid violet 19, Acid magneta	42685	586
Ponceau 2R	Acid red 26, Ponceau de xylidine	16150	480
Ponceau 3R	Acid red 18	16155	494
Azocarmine B	Acid red 103	50085	682
Azofuchsin	Acid red 33	16550	467
Azophloxine	Acid red 1, Amido naphthol red	18050	509
Biebrich scarlet	Acid red 66, Ponceau B	26905	556
Congo red	Direct red 28, Direct red Y	22120	697
Crystal ponceau 6R	Acid red 44	16250	502
Direct garnet	Direct red 10, Congo Corinth	22145	698
Azo eosin	Acid red 4	14710	380
Lissamine red 3GX	Acid red 57, Propalan red 3 GX	17053	527
Sirius	Direct red 80, Chlorantine fast red	35780	1373
Methyl blue	Aniline blue, Acid blue 93, Cotton blue	42780	800
Alkali blue		42750	574
Durazol brilliant blue	Direct blue, Direct blue 109	51310	1012
Naphthol blue black	Amido black 10B, Acid black 1	20470	617
Pontamine sky blue 6BX	Chicago blue 6B, Direct blue 1	24410	993
Isamine blue	Direct blue 41, Pyrrol blue	42700	786
Azocarmine G	Acid red 101	50085	580

available to react with the trichrome dyes. Treatment of formaldehyde-fixed tissues with picric acid, mercuric chloride solutions, or both, will enhance trichrome intensity and brilliance. Lendrum et al. (1962) recommended 'degreasing' sections for 24–48 hours in trichloroethylene prior to staining to further improve the intensity of the staining reactions. Zenker's solution, formal mercury, Bouin's fixative or picro-mercuric alcohol are the most satisfactory fixatives for trichrome techniques. The staining reactions following these fixatives will be much brighter and more saturated than formaldehyde fixation. It must be noted that mercury-containing fixatives are now rarely used due to their highly toxic nature.

van Gieson technique (van Gieson, 1889)

Sections

Paraffin. For double embedding in celloidin or low-viscosity nitrocellucose (LVN) sections, see Notes d and e below.

van Gieson solution

Saturated aqueous picric acid solution	50 ml
1% aqueous acid fuchsin solution	9 ml
Distilled water	50 ml

Method

1. Deparaffinize sections and take to water.
2. Stain nuclei by the celestine blue-hematoxylin sequence.
3. Wash in tap water.
4. Differentiate in acid alcohol.
5. Wash well in tap water.
6. Stain in van Gieson solution for 3 minutes.
7. Blot and dehydrate quickly through ascending grades of alcohol.
8. Clear in xylene and mount in permanent mounting medium.

Results

Nuclei	blue/black
Collagen	red
Other tissues	yellow

Notes

a. Fixation is not critical, buffered formalin is satisfactory.
b. Washing in water after van Gieson solution should be avoided as the color balance is impaired by this.
c. Nuclear staining should be intense before application of van Gieson solution; the picric acid will act as a differentiator.
d. Celloidin sections are washed in distilled water after van Gieson solution.
e. Celestine blue may stain the celloidin intensely. In such circumstances, Weigert's iron hematoxylin should be used.

Role of phosphotungstic and phosphomolybdic acids (PTA and PMA)

Although these acids do not give identical reactions in trichrome staining techniques, their properties are similar. Experimental work indicates that the principles involved are identical but that the two substances are not interchangeable in any given technique. Everett and Miller (1974) have shown that treatment of formalin-fixed sections with PMA or PTA reduces staining of all tissue components except collagen fibers with aniline blue and other similar anionic dyes. Using smaller dye molecules, e.g. Biebrich scarlet, blocking is less complete. These workers postulated that the differential staining by the trichrome methods occur by binding of aniline blue to basic residues in the connective tissues not already blocked by PTA. Baker (1958) stated that PMA acts as a 'colorless acid dye' of large molecular size and hence diffuses slowly.

Practical uses of PMA and PTA

In trichrome staining there are three stages at which PMA or PTA may be used. Firstly, before treatment with the small molecule dye, secondly combined in solution with the small molecule dye and thirdly before treatment with the large molecule dye. Any combination of these techniques is possible. If a section is first treated with PMA or PTA solution and then with a low concentration of a 'leveling' dye in the same solution, the leveling dye will only color the erythrocytes. A levelling dye is a small to medium size dye which is equally distributed throughout the tissue but easy to wash out. In practice, the first treatment with the PMA or PTA is frequently omitted without detriment to the final results. When a section is first treated with a leveling dye or other suitable small molecule anionic dye and then with PMA or PTA solution, the PMA or PTA competes with the dye and gains access to the collagen easily, expelling the dye in the process. If treatment is stopped at the right moment, only collagen will be free to stain when treated with a 'milling' or other large molecule dye. A milling dye is a large dye which is difficult to remove from the tissue. If treatment with the large molecule dye is greatly prolonged, staining of muscle and cytoplasm may take place.

Masson trichrome technique (Masson, 1929)

Fixation

Formal sublimate or formal saline.

Sections

All types.

Preparation of solutions

Solution a

Acid fuchsin	0.5 g
Glacial acetic acid	0.5 ml
Distilled water	100 ml

Solution b

Phosphomolybdic acid	1 g
Distilled water	100 ml

Solution c

Methyl blue	2 g
Glacial acetic acid	2.5 ml
Distilled water	100 ml

Method

1. Deparaffinize sections and take to water.
2. Remove mercury pigment by iodine, sodium thiosulfate sequence.
3. Wash in tap water.
4. Stain nuclei by the celestine blue-hematoxylin method (p. 131).
5. Differentiate with 1% acid alcohol.
6. Wash well in tap water.
7. Stain in acid fuchsin solution **a** for 5 minutes.
8. Rinse in distilled water.
9. Treat with phosphomolybdic acid solution **b** for 5 minutes.
10. Drain.
11. Stain with methyl blue solution **c** for 2–5 minutes.
12. Rinse in distilled water.
13. Treat with 1% acetic acid for 2 minutes.
14. Dehydrate through ascending grades of alcohol.
15. Clear in xylene, mount in permanent mounting medium.

Results

Nuclei	blue/black
Cytoplasm, muscle, and erythrocytes	red
Collagen	blue

Notes

a. The celestin blue-hematoxylin sequence provides a satisfactory alternative to the iron alum hematoxylin used in the original method.
b. Light green may be substituted for methyl blue.

Heidenhain's 'Azan'

This technique is not recommended as a general connective tissue method due to prolonged staining times. It may be useful in the demonstration of 'wire loop lesions' in the diagnosis of lupus nephritis in renal biopsies.

The demonstration of fibrin

Techniques for the selective demonstration of fibrin are of three types:

- Gram-Weigert
- Phosphotungstic acid-hematoxylin
- Trichrome methods.

Although there would appear to be little similarity between these methods, their selectivity depends on the use of dyes or dye complexes of suitable molecular size. Masson type trichrome techniques may be satisfactory for the demonstration of fibrin, although older deposits tend to stain as collagen. Lendrum et al. (1962) showed that modifications of the Masson technique enable older deposits of fibrin to be demonstrated. The main feature of the Martius scarlet blue (MSB) technique is the use of a small molecule yellow dye with PTA in alcoholic solution to selectively stain red cells. Early fibrin deposits may be stained by this dye, although the PTA blocks the staining of muscle, collagen and most connective tissue fibers. Following treatment with the medium sized molecule red dye, muscle and mature fibrin are stained but staining of collagen is prevented by PTA remaining from the first stage. Further treatment with aqueous PTA removes any trace of red from the collagen fibers. Final treatment with a large molecule blue dye demonstrates collagen and old fibrin deposits.

MSB technique for fibrin (Lendrum et al., 1962)

The standard MSB technique employs Martius yellow (acid yellow 24) CI 10315, brilliant crystal scarlet (acid red 44) CI 16250, and soluble or methyl blue (acid blue 93) CI 42780.

Preparation of solutions

Martius yellow solution

Martius yellow	0.5 g
PTA	2 g
95% alcohol	100 ml

Martius yellow is dissolved in alcohol before adding the phosphotungstic acid. A satisfactory

substitute for Martius yellow is the larger molecule dye, lissamine fast yellow, which has the advantage of being less easily removed by the subsequent red dye.

Brilliant crystal scarlet solution

Brilliant crystal scarlet	1 g
Glacial acetic acid	2 ml
Distilled water	100 ml

A number of medium sized molecule anionic red dyes may be substituted for the brilliant crystal scarlet, e.g. Ponceau de xylidine and azofuchsin.

PTA solution

PTA	1 g
Distilled water	100 ml

Methyl blue solution

Methyl blue	0.5 g
Glacial acetic acid	1 ml
Distilled water	100 ml

Many large molecule blue or green dyes may be substituted for the methyl blue e.g. durazol blue, pontamine sky blue, fast green FCF or naphthalene black 10B. The replacement of methyl blue by pontamine sky blue reduces the tendency of fibrin coloring by the blue dye due to the larger molecular size.

1% acetic acid

Glacial acetic acid	1 ml
Distilled water	100 ml

Method

1. Deparaffinize sections and take to water.
2. Remove mercury pigment with iodine, sodium thiosulfate treatment.
3. Stain nuclei by the celestine blue-hematoxylin sequence (p. 131).
4. Differentiate in 1% acid alcohol.
5. Wash well in tap water.
6. Rinse in 95% alcohol.
7. Stain in Martius yellow solution for 2 minutes.
8. Rinse in distilled water.
9. Stain in brilliant crystal scarlet solution for 10 minutes.
10. Rinse in distilled water.
11. Treat with PTA solution until no red remains in the collagen.
12. Rinse in distilled water.
13. Stain in methyl blue solution until collagen is sufficiently stained.
14. Rinse in 1% acetic acid.
15. Dehydrate through ascending grades of alcohol.
16. Clear in xylene and mount in permanent mounting medium.

Results

Nuclei	blue
Erythrocytes	yellow
Muscle	red
Collagen	blue
Fibrin	red (early fibrin may stain yellow and old fibrin, blue)

Notes

a. Step 11 may take up to 10 minutes although sufficient selectivity may be achieved by using a standard time of 5 minutes.

b. Step 13, examine after 2 minutes and at successive 2 minute intervals as excessive stain cannot be removed easily.

Demonstration of muscle striations

H&E and trichrome methods demonstrate muscle striations, but better definition is seen using Heidenhain's iron hematoxylin and Mallory's PTA hematoxylin.

Staining of elastic tissue fibers

Numerous techniques have evolved for the demonstration of elastic tissue fibers. Few are in current use but the most popular are Verhöeff's method, the orcein technique, Weigert's resorcin-fuchsin and the aldehyde fuchsin method. Additionally, the dyes chlorazol black E and luxol fast blue are used as techniques to determine the mechanism of elastic tissue staining.

General notes on the mechanism of elastic staining

Elastic fibers will stain intensely, but not always selectively, by a number of unrelated techniques e.g. H&E, hematoxylin-phloxine-saffron, Congo red, PAS, Verhöeff's hematoxylin, resorcin-fuchsin, aldehyde fuchsin and Taenzer-Unna orcein. The reaction of elastic fibers with eosin, phloxine or Congo red may be attributed to coulombic reactions between elastin protein and the acid dyes. The positive

PAS reaction seen in immature, fine fibers may be accounted for by the presence of carbohydrate-containing glycoproteins associated with the elastic fiber microfibrillar protein, a major component of young elastic fibers.

Verhöeff's method is the classical method for elastic fibers and works well after all routine fixatives. Coarse fibers are intensely stained but the staining of fine fibers may be poor. The differentiation step is critical to the success of this method and expertise is necessary to achieve reproducible results – it is easy to over-differentiate and lose the finer fibers. Some older texts state that the prepared working solution has a usable life of only 2–3 hours but satisfactory results can be obtained using solutions up to 48 hours old.

Verhöeff's method for elastic fibers (Verhöeff, 1908)

Preparation of stain

Solution a

Hematoxylin	5 g
Absolute alcohol	100 ml

Solution b

Ferric chloride	10 g
Distilled water	100 ml

Solution c, Lugol's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	100 ml

Verhöeff's solution

Solution **a**	20 ml
Solution **b**	8 ml
Solution **c**	8 ml

Add in the above order, mixing between additions.

Method

1. Deparaffinize sections and take to water.
2. Stain in Verhöeff's solution for15–30 minutes.
3. Rinse in water.
4. Differentiate in 2% aqueous ferric chloride until elastic tissue fibers appear black on a gray background.
5. Rinse in water.
6. Rinse in 95% alcohol to remove any staining due to iodine alone.
7. Counterstain as desired, van Gieson is conventional, although eosin may be used.
8. Blot to remove excess stain.
9. Dehydrate rapidly through ascending grades of alcohol.
10. Clear in xylene and mount in permanent mounting medium.

Results

Elastic tissue fibers	black
Other tissues according to counterstain	

Notes

a. Pretreatment with 1% potassium permanganate for 5 minutes followed by oxalic acid improves sharpness and intensity of staining.
b. Removal of mercury pigment is unnecessary as this is done by the iodine in the staining solution.
c. Rinsing in warm tap water improves intense staining of fibers.

Orcein methods

Orcein is a naturally occurring vegetable dye which has now been synthesized. Variations between batches of dye may produce erratic results with insufficient depth of stain. The main advantages of this stain are the simplicity of preparation and the choice of fixative appears unimportant.

Weigert's resorcin-fuchsin method

The standard Weigert technique employs basic fuchsin, but a number of related cationic dyes of the triphenyl-methane group may be substituted. The composition of basic fuchsin itself is variable as at least three dyes are present in many batches. These variations considerably affect the staining and storage properties of the prepared solution, as well as the stain imparted to elastic fibers. The impurities in the ferric chloride are another variable, even when fresh it contains the ferrous salt which does not produce a satisfactory staining solution. Ferric nitrate has been found to be consistently free from the ferrous salt and should be substituted for the chloride in the Weigert technique and its variations.

Mechanism of Weigert elastin staining

It has been shown that acetylation, sulfation and phosphorylation of tissues induce binding of resorcin-fuchsin to glycogen, basement membranes, reticular fibers, collagen and other tissue structures containing polysaccharides. These structures are unstained by resorcin-fuchsin without prior treatment, indicating that this binding is due to the introduction of ester groups. Extraction procedures designed to remove dyes bonded by salt or ionic linkages indicate that non-ionic bonds are involved.

Resorcin-fuchsin method (Weigert, 1898)

Preparation of Weigert resorcin-fuchsin

Add 1 g of basic fuchsin and 2 g of resorcin to 100 ml of distilled water. Boil and add 12.5 ml of freshly prepared 30% ferric chloride solution (see previous note on the use of ferric nitrate). Continue boiling for 5 minutes. Cool and filter, discarding the filtrate. Dissolve the whole of the precipitate in 100 ml of 95% ethanol using a hot plate or water bath for controlled heating and add 2 ml of concentrated HCl.

For an improved solvent dissolve the precipitate in:

2-methoxyethanol	50 ml
Distilled water	50 ml
Concentrated HCl	2 ml

Note, the staining time is reduced with this solvent.

Method

1. Deparaffinize sections and take to alcohol.
2. Place in staining solution in a Coplin jar for 1–3 hours at room temperature or 1 hour at 56°C.
3. Rinse in tap water.
4. Remove background staining by treating with 1% acid alcohol.
5. Rinse in tap water.
6. Counterstain as desired, van Gieson, eosin or trichrome methods are applicable.
7. Dehydrate through ascending grades of alcohol.
8. Clear in xylene and mount in permanent mounting medium.

Notes

a. Staining may be carried out after most fixatives. Those containing chromium salts produce less intense and more diffuse staining.

b. Acid alcohol treatment for removal of background staining can take just a few seconds but may be prolonged without harm.

c. Treatment before stage 2 for 5 minutes with 1% potassium permanganate followed by oxalic acid improves the staining.

Results

Elastic tissue fibers	brown to purple

Modifications of the Weigert technique

Hart's modification is recommended for use after fixatives containing potassium dichromate. This staining solution is simply prepared by making a dilution of between 5 and 20% of the Weigert solution in 70% alcohol containing 1% HCl. Staining time must be increased to overnight.

Sheridan's resorcin-crystal violet method substitutes 1 g of crystal violet and 1 g dextrin for the basic fuchsin in the Weigert technique.

The methyl violet/ethyl violet-resorcin method for elastic fibers (see below) has replaced the dahlia elastic tissue stain given in earlier editions as dahlia has been withdrawn from the commercial market.

Methyl violet/ethyl violet-resorcin method for elastic fibers

Preparation of staining solution

Dissolve 0.5 g of methyl violet (CI 42535) and 0.5 g of ethyl violet (CI 42600) in 100 ml of boiling distilled water. Add 2 g of resorcin and 25 ml of 30% ferric nitrate solution, continue boiling for an additional 3 minutes. Cool and filter. Discard the filtrate and dissolve the whole of the precipitate by gentle heating on hot plate or water bath in the following solution:

2 methoxyethanol	50 ml
Distilled water	50 ml
Concentrated HCl	2 ml

Preparation of the staining solution may be speeded by the use of a microwave oven at both heating stages.

Method

The same technique as for the Weigert method is employed, using the potassium permanganate and oxalic acid pretreatment. Staining may be adequate with formalin-fixed tissues in 15 minutes at room temperature.

Results

Elastic tissue fibers	blue-black

Aldehyde fuchsin

This was first introduced into histology as an elastic tissue stain by Gomori (1950). Coarse and fine fibers are stained adequately with a suitably ripened solution, although a number of other tissue components are equally well stained e.g. β cell granules of pancreas and sulfated mucosubstances. Prior oxidation in periodic acid, peracetic acid or potassium permanganate demonstrates other components, e.g. glycogen and neutral mucosubstances, but with increased intensity of elastic tissue staining.

Aldehyde fuchsin method for elastic fibers

Preparation of staining solution

Dissolve 1 g of basic fuchsin in 100 ml of 70% ethanol, heat may be used to speed the process. After cooling and filtering, add 1 ml of concentrated HCl and 2 ml of paraldehyde. Stand at room temperature for 2 days to complete the ripening process which is indicated by a color change from red to purple. Ripening time may be reduced by increasing the temperature to 50–60°C. The ripened solution should be refrigerated for storage. Batches of basic fuchsin suitable for the production of Schiff's reagent are satisfactory for the preparation of aldehyde fuchsin. Paraldehyde may lose some potency upon storage but this may be partially compensated for by the addition of an extra 0.5 ml. The staining potential of aldehyde fuchsin is greatest between 2 and 4 days after preparation, but may be adequate for the demonstration of elastic tissue fibers for several weeks if stored at 4°C.

Method

1. Deparaffinize sections and take to water.
2. Oxidize in 1% potassium permanganate for 5 minutes.
3. Rinse in tap water.
4. Remove permanganate staining by treatment with 1% oxalic acid.
5. Rinse in tap water.
6. Rinse in 70% ethanol.
7. Place in sealed container of aldehyde fuchsin for 15 minutes.
8. Rinse well in 70% ethanol.
9. Rinse in tap water.
10. Counterstain as desired, eosin, van Gieson or neutral red are suitable.
11. Dehydrate through ascending grades of alcohol.
12. Clear in xylene and mount in permanent mounting solution.

Results

Elastic tissue fibers	blue-purple

β cell granules of pancreas, some mucosubstances, some fungi, cartilage matrix and mast cell granules are also stained. Other tissues stain according to the counterstain used.

Note

Contrast between the collagen and fine elastic tissue fibers may be inadequate following van Gieson counterstain and a purely nuclear counterstain may be considered more suitable.

The demonstration of reticular fibers

These stains may be divided into those using dyes as a means of staining, and the metal impregnation methods. Dye techniques for reticular demonstration cannot be considered completely reliable as the density of the stain is insufficient to resolve the fine fibers. Staining techniques do not readily differentiate between collagen and reticular fibers. Metal impregnation techniques provide contrast enabling even the finest fibers to be seen (Fig. 12.2).

Metal impregnation techniques

Many of these have been published and most employ silver salts. The composition of these solutions vary widely, but in all the silver is in alkaline solution in a state readily able to precipitate as metallic silver. Reticular fibers have a low natural affinity for silver salts and require suitable pretreatment in order to enhance the selectivity of the impregnation. Pretreatment baths are frequently heavy metal salt solutions, commonly ferric ammonium sulfate. Treatment with silver solutions have a twofold effect. Submicroscopic sensitized sites of silver, in reduced form, are created on the reticular fibers and a considerable quantity of silver is taken up by tissues in unreduced form. These reactions are both optimized at around pH 9.0 and are rapid.

Silver taken up by the tissue in unreduced form is converted to metallic silver which is deposited

at the sensitized sites when treated with a reducing agent. Any remaining unreacted silver may be removed by treatment with sodium thiosulfate solution. A permanent preparation is produced if the silver is partially converted to a gold impregnation by treatment with gold chloride solution. This also increases the contrast. Ammoniacal silver salts in the dry state are potentially dangerous due to their explosive properties and they should be stored in solution in black plastic rather than glass bottles.

Preparation of silver solutions

These all require the solvent to be glass distilled or deionized water to prevent precipitation of insoluble silver salts. There are many formulae for suitable silver solutions but the majority follow the same pattern. A carbonate or hydroxide solution is added to a solution of silver nitrate to produce a precipitate. The precipitate is re-dissolved, usually by the addition of ammonia solution. The different formulae all require great attention to detail, glassware must be perfectly clean and solutions prepared from the purest of reagents with weights and volumes accurately measured. Any excess of ammonia in the solution results in a loss of sensitivity, giving only weak or complete lack of reticular fiber impregnation. The most reliable procedure is to add less ammonia than is needed to dissolve the precipitate and filter to remove remaining turbidity. Alternatively, back titration with silver nitrate may be employed to react with any excess ammonia.

Gordon & Sweets' method for reticular fibers (Gordon & Sweets, 1936)

Preparation of silver solution

To 5 ml of 10% aqueous silver nitrate solution add concentrated ammonia drop by drop, until the precipitate first formed dissolves, taking care to avoid any excess of ammonia. Add 5 ml of 3% sodium hydroxide solution. Re-dissolve the precipitate by the addition of concentrated ammonia drop by drop, until the solution retains a trace of opalescence. If at this stage any excess of ammonia is present, indicated by the absence of opalescence, add a few drops of 10% silver nitrate solution to produce a light precipitate. Make the volume up to 50 ml with distilled water. Filter before use. Store in a dark plastic bottle.

Method

1. Deparaffinize sections and take to water.
2. Treat with 1% potassium permanganate solution for 5 minutes.
3. Rinse in tap water.
4. Bleach in 1% oxalic acid solution.
5. Rinse in tap water.
6. Treat with 2.5% iron alum solution for at least 15 minutes.
7. Wash well in several changes of distilled water.
8. Place in a Coplin jar of silver solution for 2 minutes.
9. Rinse in several changes of distilled water.
10. Reduce in 10% aqueous formalin solution for 2 minutes.
11. Rinse in tap water.
12. Tone in 0.2% gold chloride solution for 3 minutes.
13. Rinse in tap water.
14. Treat with 5% sodium thiosulfate solution for 3 minutes.
15. Rinse in tap water.
16. Counterstain as desired.
17. Dehydrate through ascending grades of alcohol.
18. Clear in xylene and mount in permanent mounting medium.

Results

Reticular fibers	black
Nuclei	black or unstained (see Note a below)

Other elements according to counterstain

Notes

a. A short treatment with iron alum solution of less than 5 minutes gives less staining of nuclei.

b. The use of a Coplin jar for the silver solution greatly reduces the possibility of precipitation on the slide. Sections can be counterstained with eosin, nuclear fast red, tartrazine or van Gieson.

Gomori's method for reticular fibers (Gomori, 1937)

Preparation of silver solution

Add 40 ml of 10% silver nitrate solution to 10 ml of 10% potassium hydroxide solution. Allow the precipitate to settle and decant the supernatant. Wash the precipitate several times with distilled water. Add concentrated ammonia drop by drop until the precipitate has just dissolved. Add further 10% silver nitrate solution until a little precipitate remains. Dilute to 100 ml and filter. Store in a dark plastic bottle.

Method

1. Deparaffinize sections and take to water.
2. Treat with 1% potassium permanganate solution for 2 minutes.
3. Rinse in tap water.
4. Bleach in 2% potassium metabisulfate solution.
5. Rinse in tap water.
6. Treat with 2% iron alum for 2 minutes.
7. Wash in several changes of distilled water.
8. Place in Coplin jar of silver solution for 1 minute.
9. Wash in several changes of distilled water.
10. Reduce in 4% aqueous formalin solution for 3 minutes.
11. Rinse in tap water.
12. Tone in 0.2% gold chloride solution for 10 minutes.
13. Rinse in tap water.
14. Treat with 2% potassium metabisulfate solution for 1 minute.
15. Rinse in tap water.
16. Treat with 2% sodium thiosulfate solution for 1 minute.
17. Rinse in tap water.
18. Counterstain as desired, van Gieson or eosin is suitable.
19. Dehydrate through ascending grades of alcohol.
20. Clear in xylene and mount in permanent mounting medium.

Results

Reticular fibers	black
Nuclei	gray
Other tissues according to counterstain	

Russell modification of the Movat pentachrome stain (Carson, 1997) (Fig. 12.5)

Fixation

10% neutral buffered formalin or acetic formalin sublimate (4 g mercuric chloride; 20 ml of 37–40% formaldehyde; 80 ml distilled water; 5 ml glacial acetic acid).

Sections

5 µm.

Solutions

1% alcian blue solution

Alcian blue, 8 GS	1 g
Distilled water	100 ml
Glacial acetic acid	2 ml

Mix well and store at room temperature.

Alkaline alcohol

Ammonium hydroxide	10 ml
95% alcohol	90 ml

Iodine solution

Iodine	2 g
Potassium iodide	2 g
Distilled water	100 ml

Add the iodine and potassium iodide to about 25 ml of distilled water and mix until dissolved and add the remaining water.

10% absolute alcoholic hematoxylin

Hematoxylin	10 g
Absolute alcohol	100 ml

Mix until dissolved. Cap tightly and store at room temperature.

10% ferric chloride

Ferric chloride	10 g
Distilled water	100 ml

Mix until dissolved and store at room temperature.

Hematoxylin solution

10% absolute alcoholic hematoxylin	25 ml
Absolute alcohol	25 ml
10% aqueous ferric chloride	25 ml
Iodine solution	25 ml

Prepare just before use.

2% ferric chloride (for differentiation)

10% ferric chloride	10 ml
Distilled water	40 ml

Prepare just before use.

5% sodium thiosulfate

Sodium thiosulfate	5 g
Distilled water	100 ml

Mix until dissolved and store at room temperature.

Crocein scarlet-acid fuchsin

Solution a

Crocein scarlet	0.1 g
Distilled water	99.5 ml
Glacial acetic acid	0.5 ml

Mix until dissolved and store at room temperature.

Solution b

Acid fuchsin	0.1 g
Distilled water	99.5 ml
Glacial acetic acid	0.5 ml

Working solution

Solution **a**	8 parts
Solution **b**	2 parts

Prepare just before use.

5% aqueous PTA solution

PTA	5 g
Distilled water	100 ml

Mix until dissolved and store at room temperature.

Alcoholic safran

Safran du Gatinais	6 g
Absolute alcohol	100 ml

Keep tightly closed to prevent hydration.

Method

1. Deparaffinize and take to distilled water.
2. Stain in alcian blue for 20 minutes.
3. Wash in running tap water for 5 minutes.
4. Place slides in alkaline alcohol for 1 hour.
5. Wash in running tap water for 10 minutes.
6. Rinse in distilled water.
7. Stain in hematoxylin solution for 15 minutes.
8. Rinse in several changes of distilled water.
9. Differentiate in 2% aqueous ferric chloride until the elastic fibers contrast sharply with the background.
10. Rinse in distilled water.
11. Place slides in 5% sodium thiosulfate for 1 minute.
12. Wash in running tap water for 5 minutes and rinse in distilled water.
13. Stain in crocein scarlet-acid fuchsin for 1–5 minutes.
14. Rinse in several changes of distilled water.
15. Rinse in 0.5% acetic acid water.
16. Place slides in 5% aqueous PTA, two changes of 5 minutes each.
17. Rinse in 0.5% acetic acid water.
18. Rinse in three changes of absolute alcohol.
19. Stain in alcoholic safran solution for 15 minutes.
20. Rinse in three changes of absolute alcohol.
21. Clear in two or three changes of xylene and mount with a synthetic resin.

Results

Nuclei and elastic fibers	black
Collagen and reticular fibers	yellow
Ground substance, mucin	blue
Fibrinoid, fibrin	intense red
Muscle	red

Notes

The differentiation of the elastic fibers is usually complete in 2–3 minutes. The complete removal of alkaline alcohol with running water is important. Failure to remove all of the alkaline alcohol will inhibit the subsequent staining steps. This stain may be used to demonstrate *Cryptococcus neoformans*, staining the organism a brilliant blue.

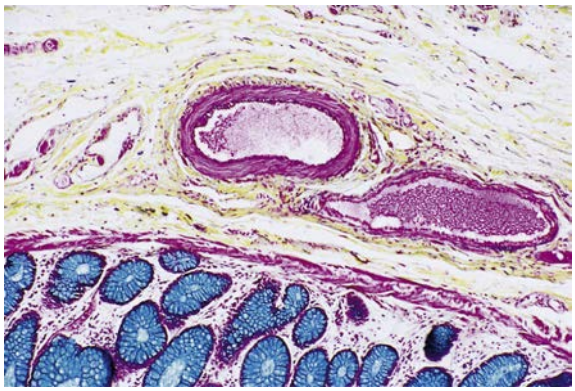

Fig. 12.5 Movat pentachrome method demonstrating fibrin, collagen and muscle in a small intestine section.

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13 Carbohydrates

Christopher Layton • John D. Bancroft

Introduction

This is a large group of compounds with the general formula $C_n(H_2O)_n$. The role of carbohydrates in cellular metabolism has been known for many years but carbohydrates have more recently been implicated in a wide range of cellular functions including protein folding, cell adhesion, enzyme activity and immune recognition (Varki et al., 1999). Histochemical techniques for the detection and characterization of carbohydrates and carbohydrate-containing macromolecules (glycoconjugates) are used regularly in the histology laboratory. These techniques may provide invaluable information assisting the pathologist in diagnosing and characterizing various pathological conditions including neoplasia, inflammation, autoimmune and genetic disorders, and infectious diseases.

Classification of carbohydrates

Carbohydrates are divided into two broad categories. Firstly, simple carbohydrates, molecules composed purely of carbohydrates, and secondly glycoconjugates composed of carbohydrates and other molecules such as protein or lipid. These are further categorized as in Table 13.1. Although lipid carbohydrate complexes are widely distributed in cells and tissues, these types of molecule are not discussed here as they are not detectable by the routine histochemical techniques described in this chapter.

Monosaccharides

These are the simplest form of a carbohydrate. Typical monosaccharides have the empirical formula $(CH_2O)_n$, where n is a value between 3 and 9. Glucose (Fig. 13.1) is a six-carbon simple carbohydrate which is not charged or ionized and referred to as a neutral sugar. Other neutral sugars include mannose, galactose and fructose. Monosaccharides contain asymmetric carbons referred to as chiral centers and the letters D or L at the beginning of a name refer to the structure of one of the chiral carbons within the molecule. D-Monosaccharides predominate in nature.

The high numbers of hydroxyl (OH) groups present in the monosaccharide make most of them extremely water soluble. The majority of monosaccharides within a tissue specimen are lost during fixation and tissue processing due to their small size and water solubility and, as a result are not easily demonstrated by most histochemical techniques. The basic monosaccharide structure however, represents the building blocks of larger, more complex carbohydrates. The chemical and physical properties of the polysaccharides and glycoconjugates are largely determined by the types of monosaccharide which make up these molecules and the various reactive groups within them.

Polysaccharides

These are large macromolecules composed of multiple monosaccharides joined by covalent bonds known as glycosidic linkages. Fig. 13.2 demonstrates an α1–4 glycosidic linkage connecting two units of glucose which is the predominant linkage in the polysaccharides starch and glycogen. Additionally, some of the glucose units of these polysaccharides may have more than one glycosidic linkage, forming a branching structure resembling a tree. Starch and glycogen are large macromolecules with molecular weights exceeding 1×10^6 Daltons. They consist of

Table 13.1 Basic classification of carbohydrates and glycoconjugates	
SIMPLE CARBOHYDRATES	
Monosaccharides	glucose mannose galactose
Oligosaccharides	sucrose maltose
Polysaccharides	glycogen starch
GLYCOCONJUGATES	
Connective tissue glycoconjugates	proteoglycans hyaluronic acid
Mucins	neutral mucins sialomucins sulfomucins
Other glycoproteins	membrane proteins (receptors, cell adhesion molecules) blood group antigens
Glycolipids	cerebrosides gangliosides

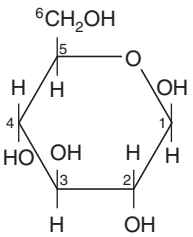

Fig. 13.1 Structure of β-D-glucose.

glucose units with α1–4 and α1–6 glycosidic linkages, but differ in size and branching structure.

Glycogen is the only polysaccharide found in animals which is frequently evaluated by histochemical techniques. It serves as a major form of stored energy reserves in humans. Carbohydrates absorbed following a meal are converted to glycogen by hepatocytes in the liver. During fasting, glycogen is broken down into glucose units which can be used as an immediate source of energy. Glycogen occupies a significant volume of the cytoplasm of hepatocytes and can form intranuclear inclusions. Skeletal and cardiac muscle cells also store significant quantities of glycogen.

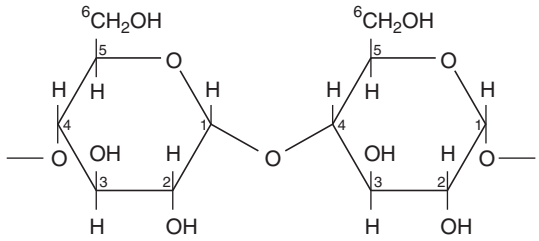

Fig. 13.2 Structure of two glucose units joined by an α1–4 glycosidic linkage.

There are a number of pathological conditions in which the histochemical assessment of glycogen content or accumulation may be of value diagnostically (Table 13.2). Glycogen storage diseases in humans are the result of inherited defects of one or more of the enzymes involved in the synthesis or breakdown of glycogen (Cori & Cori, 1952; Hers, 1963). In most of these disorders, the liver shows massive accumulation of glycogen but this can also occur in skeletal and cardiac muscle.

Connective tissue glycoconjugates, the proteoglycans

These are also referred to as connective tissue mucins or mucopolysaccharides. They are large glycoconjugate complexes which are found in high concentrations within the extracellular matrix of connective tissues. Proteoglycans are highly glycosylated and, in many cases, 90–95% of the molecular weight of a typical proteoglycan is the carbohydrate component.

The carbohydrate components of proteoglycans are known as glycosaminoglycans which are large polysaccharide polymers covalently bound to the protein core. The typical glycosaminoglycan is a long unbranched polysaccharide of repeating disaccharide units each made up of two different monosaccharides. Each disaccharide is typically composed of a carboxylated uronic acid, glucuronic or iduronic, and a hexosamine, e.g. *N*-acetylglucosamine or *N*-acetylgalactosamine. The hexosamines frequently contain highly acidic sulfate groups. There are six distinct classes of glycosaminoglycans (Table 13.3),

Table 13.2 Summary of the different types of carbohydrates and glycoconjugates and their diagnostic significance

Type	Location	Function	Associated pathological condition
Glycogen	Liver, skeletal muscle, cardiac muscle, hair follicles, cervical epithelium	Storage form of carbohydrate	Found in a wide range of malignancies, e.g. Ewing's sarcoma, rhabdomyosarcoma, seminoma. Abnormal accumulation in tissues of patients with glycogen storage diseases
Proteoglycans and hyaluronic acid	Cartilage, heart valves, blood vessels, tendons, ligaments, extracellular matrices, and ubiquitously expressed on the membranes of many cell types	Support, lubrication, cell adhesion	Found in certain sarcomas, e.g. myxoid chondrosarcomas, myxoid liposarcomas, myxoid fibrous histiocytomas. Abnormal accumulation in tissues of patients with mucopolysaccharidoses
Mucins	Epithelia of the gastrointestinal tract, respiratory tract, reproductive tract	Secreted mucins – lubrication and protection Membrane-bound mucins – cell adhesion and regulation of proliferation	Frequently found in adenocarcinomas of the gastrointestinal tract. Aberrant or inappropriate expression of specific mucin types occurs frequently in the neoplastic process
Glycoproteins	Ubiquitously expressed on cell membranes Blood group antigens Secreted products such as peptide hormones and immunoglobulins	Multiple and diverse functions such as cell adhesion, immune recognition, regulation of receptor ligand binding	Aberrant expression of blood group antigens in various malignancies

Table 13.3 Characterization of glycosaminoglycans

Glycosaminoglycan	Disaccharide repeat	Location
Chondroitin sulfate[a]	Glucuronic acid and *N*-acetylgalactosamine	Cartilage, tendons, ligaments, aorta, cell membranes
Dermatan sulfate	Iduronic acid and *N*-acetylgalactosamine	Skin, blood vessels, heart valves
Keratan sulfate	Galactose and *N*-acetylglucosamine	Cartilage, cornea
Heparan sulfate[b]	Glucuronic acid and *N*-acetylglucosamine or *N*-sulfate glucosamine	Blood vessels, aorta, cell membranes
Heparin	Glucuronic acid and *N*-acetylglucosamine or *N*-sulfate glucosamine	Mast cell granules
Hyaluronic acid	Glucuronic acid and *N*-acetylglucosamine	Synovial fluid, vitreous humor, loose connective tissues Small amounts are found in cartilage where it serves as a scaffold for the proteoglycans

[a]Chondroitin sulfates may be subcategorized as chondroitin 4-sulfate or chondroitin 6-sulfate depending upon the position of the sulfate group in the *N*-acetylgalactosamine.

[b]Heparin and heparan sulfate differ structurally in the degree of sulfation of the glucosamine units. Heparin contains more sulfate and fewer *N*-acetyl groups in this unit.

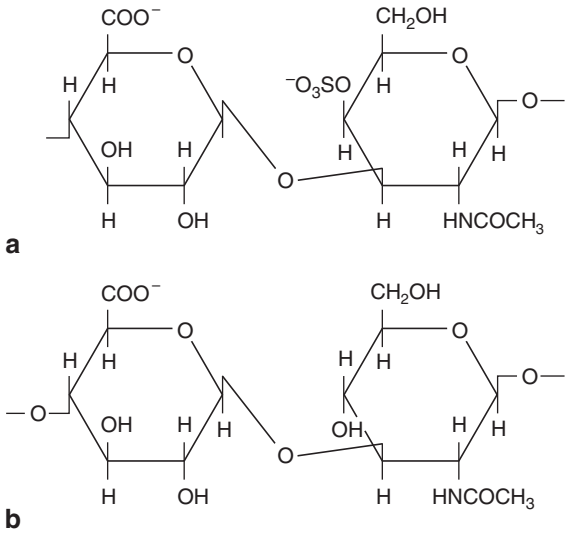

Fig. 13.3 The repeating disaccharide units of the glycosaminoglycans, chondroitin 4-sulfate (**a**) and hyaluronic acid (**b**).

chondroitin sulfates being the most abundant in humans. Fig. 13.3a illustrates the structure of chondroitin 4-sulfate.

The glycosaminoglycan chains are covalently bound to a protein core of the proteoglycan via a side chain of the amino acids serine or threonine (*O*-glycosidic linkage) and to a lesser extent to asparagine (*N*-glycosidic linkage). The number of glycosaminoglycan chains varies greatly among different proteoglycans.

Hyaluronic acid (Fig. 13.3b) is an exception to this structure as it does not contain a covalently bound protein core (Mason et al., 1982). It is a polymer of repeating *N*-acetylglucosamine and glucuronic acid disaccharide units (Roden, 1980). Hyaluronic acid also differs from the other glycosaminoglycans in the absence of sulfate groups. In spite of these differences, hyaluronic acid is classified as a glycosaminoglycan because of its structural similarity. It is found in high concentrations in synovial fluid and in the ground substance and connective tissue matrices where other proteoglycans are found.

The negatively charged sulfate and/or carboxyl groups, together with numerous hydroxyl groupings make most proteoglycans extremely hydrophilic. This property accounts for the gel like consistency of the extracellular matrix and connective tissues such as cartilage. The proteoglycans in cartilage contain many glycosaminoglycan chains and are capable of binding a large volume of water. Proteoglycans act in stabilizing and supporting fibrous elements in connective tissue. Tissues which contain high concentrations of proteoglycans include cartilage, tendons, ligaments, blood vessels, heart valves and skin (Table 13.3).

Several pathological conditions involve accumulation of glycosaminoglycans or proteoglycans (Table 13.2). The mucopolysaccharidoses are a group of genetic disorders which result from a deficiency of one or more of the enzymes involved in the degradation of heparan sulfate and dermatan sulfate (McKusick & Neufeld, 1983). This causes an abnormal accumulation of glycosaminoglycans in connective tissues as well as cell types such as neurons, histiocytes and macrophages.

Glycosaminoglycans and proteoglycans are produced by a number of different sarcomas. Hyaluronic acid and chondroitin sulfates in particular may be found in high concentrations in myxoid chondrosarcomas as well as the myxoid variants of liposarcoma and malignant fibrous histiocytoma (Tighe, 1963; Kindblom & Angervall, 1975; Weiss & Goldblum, 2001). Proteoglycans may also be observed in the stromal components of sarcomas and certain carcinomas.

Mucins

These, like the proteoglycans consist of polysaccharide chains covalently linked to a protein core (Gendler & Spicer, 1995). Typically, the carbohydrate component is attached via an *O*-glycosidic linkage to serine or threonine. The serine- and threonine-rich protein core may contain anywhere from several hundred to several thousand amino acids. A defining structure of the epithelial mucins is the presence of paired repeated amino acid sequences within the protein core. Mucins are categorized numerically into functionally distinct families based in part upon differences in the paired amino acid sequences and

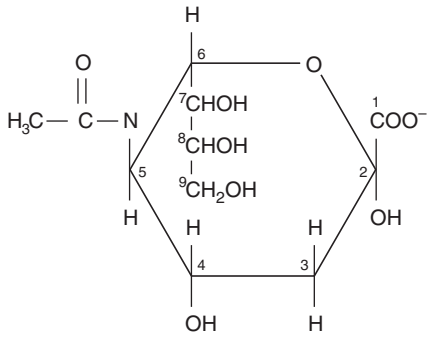

Fig. 13.4 Structure of *N*-acetylneuraminic acid, a common sialic acid in humans.

the structure of their protein core (Perez-Vilar & Hill, 1999).

The carbohydrate content of a mucin may account for up to 90% of its molecular weight. In contrast to the glycosaminoglycans which are strongly acidic polyanions, the polysaccharide chains of mucins vary from neutral or weakly acidic to strongly acidic sulfomucins. They also demonstrate a more varied composition of monosaccharide units.

Neutral mucins contain a high content of uncharged monosaccharides, e.g. mannose, galactose and galactosamine, and are found in high concentrations in the gastric mucosal epithelium, Brunner's glands in the duodenum and the prostatic epithelium.

The sialic acids (Fig. 13.4) are a diverse group of nine-carbon monosaccharides which contain a carboxylate group at the carbon in position 1 (Schauer, 1982; Varki et al., 1999). This is ionized at a physiological pH and imparts an overall negative charge on the molecule.

The function of the mucins depends on the tissue location of the mucin-producing cell and the mucin type. The secreted mucins usually provide lubrication and protection for the secreting cells and/or tissues in the immediate area. The role of the membrane-bound mucins is not fully understood. These mucins are possibly involved in the regulation of cellular functions such as cell proliferation and cell adhesion (Wesseling et al., 1995; Moniaux et al., 1999; Schroeder et al., 2001).

Whilst immunohistochemistry has largely replaced special stains in the differential diagnosis of anaplastic tumors or tumors of unknown origin, detection of mucin in a tumor may be a valuable clue in their identification. Malignancies derived from simple epithelial tissues, carcinomas, frequently contain detectable mucin whereas melanomas, lymphomas and sarcomas rarely exhibit significant levels. Additionally, determining the type of mucin, i.e. neutral or acidic may be helpful in evaluating neoplastic changes within a tissue. The detection of acid or sulfomucins within the gastric mucosa may aid the detection and characterization of intestinal metaplasia, a change associated with gastric carcinoma (Turani et al., 1986).

Other glycoproteins

There is a wide range of protein carbohydrate conjugates which are not easily categorized and fall under the general heading of glycoproteins. This is a varied group of molecules with differing carbohydrate composition, protein structure and function. These molecules frequently contain relatively short oligosaccharide or polysaccharide chains attached to a protein core. Common carbohydrate moieties include neutral sugars, e.g. mannose and *N*-acetylglucosamine. The carboxylated sialic acids may also be present.

Glycoproteins are universally expressed throughout the cells and tissues of the body with many of the proteins on the external surfaces of cell membranes containing carbohydrates. The cluster of differentiation, CD markers, found on the surface of lymphocytes frequently contain numerous glycosylation sites. These glycoproteins are involved in a variety of cellular functions, including cell adhesion and lymphocyte activation. Many of the proteins and peptides secreted from cells, e.g. cytokines, growth factors and other hormones contain glycosylation sites.

Fixation

The selection of an appropriate fixative for the histochemical detection of carbohydrates depends on the type of carbohydrate to be demonstrated. The

fixation of glycogen is more difficult than that of the glycoconjugates such as the mucins and proteoglycans. Due to the aqueous solubility of glycogen, many of the older studies recommended the avoidance of aqueous-based fixatives such as formalin (Lillie, 1954) but it is now accepted that glycogen loss during formalin fixation does not compromise the ability to detect glycogen with, e.g. the periodic acid-Schiff (PAS) method. This is due to the retention of a portion of the cellular glycogen by non-covalent association with adjacent proteins (Manns, 1958).

Whilst neutral buffered formalin (NBF) is an acceptable fixative for glycogen, the alcoholic formalins are superior fixatives for its preservation (Lillie, 1954). Rossman's fluid, alcoholic formalin with picric acid, is recommended for glycogen fixation (Bancroft & Cook, 1994) but mercuric chloride-containing fixatives such as Zenker's-acetic acid or Susa's are not advised (Manns, 1958; Bancroft & Cook, 1994).

It is essential that tissues intended for glycogen analysis should be placed in a fixative promptly following their removal, as glycogen present in many animal tissues is labile and susceptible to autolytic changes. The tissue should be refrigerated, if immediate fixation is not possible and ideally fixation should be carried out at 4°C to minimize the streaming artifact which frequently occurs in fixed tissues (Lillie, 1954).

Fixation requirements for the mucins and proteoglycans are less stringent than those for glycogen as these carbohydrates are covalently bound to proteins, and the principal effect of fixation occurs on the protein portion of the molecule. Formalin or alcoholic formalin fixation is usually adequate for preservation. The carbohydrate deposits of the mucopolysaccharidoses are less stable than typical mucins or proteoglycans (Bancroft & Cook, 1994) and fresh or frozen sections are recommended, although alcoholic formalin is also satisfactory.

Techniques for the demonstration of carbohydrates

A summary of the histochemical techniques used for the demonstration of carbohydrates and glycoconjugates and their results are outlined in Table 13.4.

The periodic acid-Schiff (PAS) technique

This is without question the most versatile and widely used technique for the demonstration of carbohydrates or glycoconjugates. The first histochemical use of this technique was by McManus (1946) for the demonstration of mucin. Subsequently other studies have demonstrated the ability of the PAS technique to demonstrate other carbohydrate-containing molecules, e.g. glycogen and certain glycoproteins (Lillie, 1947, 1951; McManus, 1948). The list of PAS-reactive tissues and cell types is long and varied and Table 13.5 is a list of PAS-reactive cells and tissue components which are frequently evaluated in the histology laboratory. This is not intended to be an all-inclusive list and the reader is directed to several references (Lillie, 1951; Thompson, 1966; Bancroft & Cook, 1994) for further reading.

The detection of mucins or glycogen by the PAS technique may aid the differential diagnosis of tumors. The reactivity of Schiff reagent with glycoproteins within the basal lamina makes the PAS technique a valuable means of assessing basement membrane thickness (Hennigar, 1987). Increased basement membrane thickness, particularly in the glomerular capillaries of the kidney, occurs in a number of pathological conditions. The PAS technique is also a sensitive and relatively fast means of demonstrating viable fungi in tissue sections due to the presence of periodic acid-reactive polysaccharides in the capsules or walls of many fungal species. Common fungal species which are PAS reactive include *Candida albicans*, *Histoplasma capsulatum*, *Cryptococcus* and *Blastomyces* (Harley, 1987).

Mechanism of the PAS technique

The PAS technique is based upon the reactivity of free aldehyde groups within carbohydrates with the Schiff reagent to form a bright red/magenta end product. The initial step in the PAS technique is the oxidation of hydroxyl groups attached to adjacent carbon atoms, 1,2-glycols, within the

Table 13.4 Summary of the specificity of the techniques for the detection of carbohydrates and glycoconjugates

					Ab 2.5-				HID-		AF-	
	PAS	PAS-D	Ab 2.5	Ab 1.0	PAS	Muc	Coll	HID	Ab 2.5	AF	Ab 2.5	Meta
POLYSACCHARIDES												
Glycogen	+	–	–	–	+(M)	–	–	–	–	–	–	–
CONN TISS GC												
Proteoglycans	–	–	+	+	+(B)	V/–	+	+	+(BB)	+	+(P)	+
Hyaluronic acid	–	–	+	–	+(B)	V/–	+	–	+(B)	–	+(B)	+
MUCINS												
Neutral	+	+	–	–	+(M)	–	–	–	–	–	–	–
Sialomucin (labile)	+	+	+	–	+(B)	+	+	–	+(B)	–	+(B)	+
Sialomucin (resist)	–	–	+	–	+(B)	+	+	–	+(B)	–	+(B)	+
Sulfomucin	V/–	V/–	+	+	+(B)	+	+	+	+(BB)	+	+(P)	+

PAS = conventional periodic acid–Schiff
D = diastase digestion
Ab 2.5 = alcian blue at pH 2.5
Ab 1.0 = alcian blue at pH 1.0
Muc = mucicarmine
Coll = colloidal iron
HID = high iron diamine
AF = aldehyde fuchsin
Meta = metachromatic techniques
Conn Tiss GC = connective tissue glycoconjugates
(labile) = digested with neuraminidase
(resist) = resistant to neuraminidase digestion
V/– = variable to negative
When two combined stains are used, the letter in parentheses represents the predominant color:
(B) = blue
(BB) = brown/black
(P) = purple
(M) = magenta

Table 13.5 PAS-reactive cells and tissue components

Glycogen
Starch
Mucin (sialomucin, neutral mucin)
Basement membranes
α-antitrypsin
Reticulin
Fungi (capsules)
Pancreatic zymogen granules
Thyroid colloid
Corpora amylacea
Russell bodies

carbohydrate. The result is the formation of two free aldehyde groups and the cleavage of the adjoining carbon-to-carbon bond (Fig. 13.5). This is produced by treatment of the sections with a dilute solution of periodic acid (HIO_4), most protocols using a 0.5–1.0% solution for 5–10 minutes. Other oxidants, e.g. chromic acid and potassium permanganate are used in variations of the technique (Bauer, 1933; Thompson, 1966) but these oxidize the aldehyde groups further, to carboxylic groups which are not reactive with Schiff reagent. As a result, the sensitivity of techniques

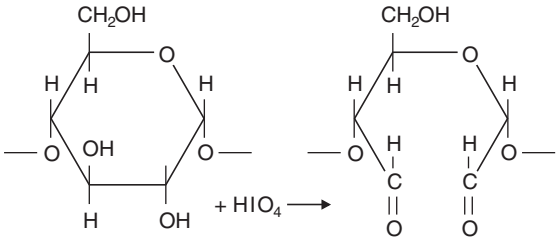

Fig. 13.5 Periodic acid (HIO_4) oxidation of a glucose unit within glycogen. Note the cleavage of the bond between carbons 2 and 3 and the formation of aldehyde groups at these carbons.

using these oxidants is less than that of the PAS technique.

The intensity of the color which develops following reaction with Schiff reagent is dependent upon the tissue concentrations of reactive glycol structures (Leblond et al., 1957). Monosaccharides which lack 1,2-glycols or contain hydroxyl groups in an ester or glycosidic linkage are not susceptible to periodic acid oxidation and hence cannot be detected with the PAS technique.

Periodic acid also oxidizes substances other than carbohydrates to form reactive aldehydes. The α-amino alcohols of serine and threonine are oxidized by periodic acid but only when present at the end of the protein chain (Thompson, 1966). It is also possible for periodic acid to oxidize hydroxylysine regardless of its position in the protein chain. However, it is doubtful whether the reactivity of these amino acids contributes significantly to the PAS reactivity in tissue sections.

Schiff reagent

This is prepared from basic fuchsin which is not a specific dye but a mixture of triarylmethane dyes such as pararosaniline, rosaniline and new fuchsin (Lillie, 1977). The individual components of basic fuchsin can also be used as starting points for the preparation of Schiff reagent.

A number of methods for the synthesis of Schiff reagent have been described since Schiff's original in 1866 but in all, an aqueous solution of sulfurous acid is produced. This may be generated from the reaction of sodium metabisulfite ($Na_2S_2O_5$) with a mineral acid, e.g. hydrochloric acid (HCl), or by the reaction of thionyl chloride ($SOCl_2$) with water (Barger & DeLamater, 1948; Longley, 1952). Sulfur dioxide is the active agent in the production of Schiff reagent and the source of the sulfur dioxide is not critical as long as the by-products from the reaction producing sulfurous acid do not interfere with the reaction with basic fuchsin (Barger & DeLamater, 1948).

The reaction of sulfur dioxide with basic fuchsin results in the addition of a sulfonic acid group to the central carbon of the triarylmethane molecule. The magenta or red color is lost due to the reduction of the quinoid configuration within the triarylmethane molecule. The free amino groups of the triarylmethane react with additional one or two equivalents of sulfur dioxide to form Schiff reagent (Lillie, 1977). As described above, Schiff reagent reacts with the free aldehydes generated from 1,2-glycol groups in periodic acid-treated carbohydrates. The initial monosaccharide-Schiff reagent conjugate is a colorless intermediate reaction. The loosely bound sulfonate of the central carbon is removed in the following aqueous rinse. The re-establishment of the quinoid structure of the triarylmethane molecule results in the deposition of a deep red/magenta coloration at the site of the carbohydrate-Schiff reagent complex (Lillie, 1977).

PAS technique (modified McManus, 1946)

Solutions

Periodic acid solution

Periodic acid	1 g
Distilled water	100 ml

Preparation of Schiff reagent

Dissolve 1 g of basic fuchsin and 1.9 g of sodium metabisulfite ($Na_2S_2O_5$) in 100 ml of 0.15 M hydrochloric acid (HCl). Shake the solution at intervals or on a mechanical shaker for 2 hours. The solution should be clear and yellow to light brown in color. Add 500 mg of activated charcoal and shake for 1 to 2 minutes. Filter the solution through a No. 1 Whatman filter into a bottle. The filtered solution should be clear and

colorless. If the solution is yellow, repeat the charcoal decolorization using a fresh lot of activated charcoal. Store at 4°C. Solution is stable for several months.

Method

1. Dewax in xylene and rehydrate through graded ethanols to distilled water.
2. Oxidize with periodic acid for 5 minutes.
3. Rinse in several changes of distilled water.
4. Cover the sections with Schiff reagent for 15 minutes.
5. Rinse in running tap water for 5–10 minutes.
6. Stain the nuclei with hematoxylin. Differentiate and blue the sections.
7. Dehydrate in graded ethanols and clear with xylene.
8. Coverslip.

Results

Glycogen, neutral/sialomucins	magenta
Various glycoproteins	magenta
Nuclei	blue

Notes

a. The intensity of stain is dependent to some extent on the length of treatment with the periodic acid and Schiff reagent. For basement membranes, a longer time in periodic acid (10 minutes) and Schiff reagent (20 minutes) may give better results.

b. Earlier descriptions of the PAS procedure frequently recommended post-Schiff bisulfite rinses for the reduction of background. This is not necessary provided the slides are adequately rinsed in tap water.

c. Fixatives containing glutaraldehyde should be avoided if tissues are to be stained with the PAS technique. Glutaraldehyde contains two aldehyde groups and tissues fixed in it contain free aldehyde groups capable of undergoing the Schiff reaction resulting in non-specific background staining.

d. Staining of glycolipids may be detected when frozen sections are used. In addition, staining of unsaturated lipids may occur in some cases due to the oxidation of carbon-to-carbon double bonds to produce Schiff-reactive aldehyde groups. However, glycolipids and unsaturated lipids rarely interfere with interpretation of results obtained from paraffin-embedded tissues as a significant loss of these molecules occurs during tissue processing.

Standard alcian blue technique

Alcian blue is a large conjugated dye molecule initially used for the dyeing of textile fibers. It is composed of a central copper-containing phthalocyanine ring linked to four isothiouronium groups via thioether bonds. The isothiouronium groups are moderately strong bases and account for the cationic nature of alcian blue (Scott et al., 1964). A variety of alcian blue dyes have been produced in the past which differ in the number of linked isothiouronium groups as well as the components of the diluent (Scott et al., 1964; Horobin & Kiernan, 2002). Alcian blue 8GX is the recommended dye for histological techniques (Scott & Mowry, 1970).

The exact mechanisms by which alcian blue stains carbohydrates are unknown, but it is widely believed that the cationic isothiouronium groups bond via electrostatic linkages with polyanionic molecules within tissues (Pearse, 1960; Quintarelli et al., 1964). The sulfate and carboxylate groups of chondroitin sulfate, dermatan sulfate, heparan sulfate and hyaluronic acid are ionized at a pH of 2.5 and therefore carry a negative charge. This accounts for the staining of the proteoglycan/hyaluronic acid components of connective tissue and cartilage with alcian blue at this pH. Similarly, the acidic epithelial mucins, e.g. the sialomucins and sulfomucins of the large intestine are reactive at pH 2.5. Neutral mucins, e.g. in the gastric mucosa and Brunner's glands are not reactive with alcian blue.

Alcian blue pH 2.5 technique (modified Mowry, 1956)

Solutions

Alcian blue solution	
Alcian blue 8GX	1 g
3% acetic acid solution	100 ml
Nuclear fast red	
Aluminum sulfate	5 g
Distilled water	100 ml
Nuclear fast red	0.1 g

Dissolve the aluminum sulfate in the water with heat. Add the nuclear fast red to water while still hot and filter.

Method

1. Dewax in xylene and rehydrate through graded ethanols to distilled water.
2. Stain in the alcian blue solution for 30 minutes.
3. Rinse in running tap water for 5 minutes.
4. Counterstain in nuclear fast red for 10 minutes.
5. Wash in running tap water for 1 minute.
6. Dehydrate in graded ethanols.
7. Clear in xylene and mount in a miscible medium.

Results

Acid mucins (sulfomucins and sialomucins)	blue
Proteoglycans and hyaluronic acid	blue
Nuclei	red

Note

To selectively identify sulfomucins and proteoglycans a low pH (pH 1) alcian blue solution should be used. Add 1.0 g alcian blue 8GX to 100 ml of 0.1 M hydrochloric acid. The staining procedure and incubation times are the same as those in the alcian blue pH 2.5 protocol.

Low pH alcian blue technique

Varying the pH of the alcian blue solution can be useful in identifying the subtypes of acidic mucins and proteoglycans present in a tissue (Spicer, 1960; Lev & Spicer, 1964; Sorvari & Sorvari, 1969). The carboxylated sialomucins and hyaluronic acid do not demonstrate the same magnitude of acidity as the sulfomucins and sulfate-containing proteoglycans, so these groups are not capable of ionization and are not charged at a pH of 1 or less. Conversely, the sulfomucins and sulfate-containing proteoglycans are ionized and negatively charged at a pH of 1. The staining observed following incubation in an alcian blue solution at a pH of 1 therefore is due predominately to sulfate groups among the mucins or proteoglycans. Examples of tissues or cell types which exhibit staining in an alcian blue solution at a pH of 1 include cartilage, goblet cell mucins of the large intestines and the mucins of bronchial serous glands.

Combined alcian blue-PAS

This combination of techniques differentiates neutral mucins from acidic mucins within a tissue section (Mowry, 1963). It is also valuable as a means of detecting mucins; a lack of staining with the combined alcian blue-PAS technique strongly suggests that the substance in question is not a mucin.

In most protocols, sections are stained with a standard alcian blue pH 2.5 method followed by PAS. The alcian blue stains sialomucins, sulfomucins, and proteoglycans blue. Neutral mucins are stained deep red/magenta with the PAS. Tissues and cells which contain both neutral and acidic mucins will stain varying shades of purple due to the binding of alcian blue and the reactivity with Schiff reagent. This is seen in the goblet cells of the small intestine which contain neutral and sialomucins (Spicer, 1960).

Combined alcian blue-PAS technique (Mowry, 1956, 1963)

Fixation

Any fixative.

Sections

Paraffin wax processed or frozen.

Solutions

Alcian blue solution (in 3% acetic acid): see above.

Periodic acid solution: see page 183.

Preparation of Schiff reagent: see page 183.

Method

1. Dewax in xylene and rehydrate through graded ethanols to distilled water.
2. Stain in the alcian blue solution for 30 minutes.
3. Rinse in running tap water for 5 minutes and then briefly in distilled water.
4. Oxidize with periodic acid for 5 minutes.
5. Rinse in running tap water for 5 minutes.
6. Cover the sections with Schiff reagent for 15 minutes.
7. Rinse in running tap water for 10 minutes.
8. Stain lightly with hematoxylin.

9. Rinse in running tap water for 5–10 minutes and blue in an appropriate blueing solution.
10. Rinse in tap water for 5 minutes.
11. Dehydrate in graded ethanols, clear with xylene, and mount with a miscible medium.

Results

Glycogen, neutral mucins, various glycoproteins	magenta
Acid mucins (sulfomucins and sialomucins)	blue
Proteoglycans and hyaluronic acid	blue

Notes

a. Cells or tissue which contain neutral mucins and acid mucins may stain various shades of blue-purple to purple.

b. It is important to stain lightly with hematoxylin in order to avoid cytoplasmic or mucin staining as this could potentially mask the color of the alcian blue.

c. Several studies have shown that the staining sequence of the combined alcian blue-PAS technique can influence the end results (Johannes & Klessen, 1984; Yamabayashi, 1987). When the PAS technique is applied prior to the alcian blue, neutral mucins and glycogen may stain purple. In contrast, these substances are colored magenta when stained with the alcian blue-PAS sequence. The reason why neutral carbohydrate moieties acquire an affinity for alcian blue following the PAS procedure is unknown but it has been suggested that the aldehyde groups generated during periodic acid-mediated oxidation may react with sulfite present in the Schiff solution to form an anionic group which subsequently may bind with alcian blue (Johannes & Klessen, 1984).

Mucicarmine

This is one of the oldest histochemical methods for the visualization of mucins in specimens (Mayer, 1896; Southgate, 1927) but with the development of newer methods, e.g. PAS, alcian blue and colloidal iron its use has declined. The technique however, remains valuable for demonstrating acidic mucins.

The mucicarmine technique is specific for mucins of epithelial origin, and like PAS and alcian blue, this technique may be useful for the identification of adenocarcinomas, particularly of the gastrointestinal tract. The capsule of the fungus *Cryptococcus neoformans* may also be detected with this technique.

Mucicarmine technique (modified Southgate, 1927)

Solutions

Southgate's mucicarmine stock solution

Carmine (alum lake)	1 g
Aluminum hydroxide	1 g
50% ethanol	100 ml

Add the above reagents to a 500 ml Pyrex flask. Shake well and add 0.5 g of anhydrous aluminum chloride. Place the flask in a boiling water bath; bring the solution to a boil. Agitate while boiling for 2.5 to 3 minutes. Cool the flask under running tap water. Filter and store at 4°C. Stable for several months.

Mucicarmine working solution

Southgate's mucicarmine stock solution	10 ml
Distilled water	90 ml

Alcoholic hematoxylin

Hematoxylin	1 g
Ethanol (95%)	100 ml

Acidified ferric chloride stock solution

Ferric chloride	2.48 g
Distilled water	97 ml
Concentrated hydrochloric acid (HCl)	1 ml

Weigert's iron hematoxylin working solution

Alcoholic hematoxylin	50 ml
Acidified ferric chloride solution	50 ml

This solution should be mixed just before use.

Metanil yellow working solution

Metanil yellow	0.25 g
Distilled water	100 ml
Glacial acetic acid	0.25 ml

Mix and store in a brown bottle or a bottle completely wrapped with aluminum foil.

Method

1. Dewax with xylene and rehydrate through graded ethanols to water.
2. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
3. Rinse in running tap water for 10 minutes.
4. Stain in the mucicarmine working solution for 30 minutes.
5. Rinse slides in two changes of distilled water.
6. Stain in the metanil yellow working solution for 30–60 seconds.
7. Rinse quickly in distilled water.
8. Dehydrate in graded ethanols and clear in xylene.
9. Coverslip using a miscible mounting medium.

Results

Acidic epithelial mucins	pink/red
Nuclei	blue/black
Other tissue components	light yellow

Note

The staining period with the mucicarmine working solution may be increased to 60 minutes if necessary.

Colloidal iron

This technique was initially described by Hale in 1946 for the detection of acid mucopolysaccharides. Numerous modifications of the original technique have been reported (Muller, 1955; Rinehart & Abul-Haj, 1951; Mowry, 1958) but all of the techniques are based upon the attraction of ferric cations in a colloidal ferric oxide solution for negatively charged carboxyl and sulfate groups of acid mucins and proteoglycans. The tissue-bound ferric ions are visualized by treatment with potassium ferrocyanide to form bright blue deposits of ferric ferrocyanide or Prussian blue.

The colloidal iron technique can also be used with a PAS procedure when sections are incubated with the colloidal iron solution and stained with potassium ferrocyanide prior to periodic acid oxidation. Acid mucins, proteoglycans and hyaluronic acid stain bright blue with the colloidal iron/potassium ferrocyanide while the neutral mucins and glycogen are colored red/magenta by the Schiff reaction.

Colloidal iron technique (modified Muller, 1955; Mowry, 1958)

Solutions

Stock colloidal iron solution

Bring 250 ml of distilled water to a boil and add 4.4 ml of a 29% ferric chloride solution (USP XI). Continue to boil until the solution turns dark red, at which time the solution should be removed from the heat and allowed to cool. This solution is stable for one year.

Colloidal iron working solution

Stock colloidal iron solution	20 ml
Distilled water	15 ml
Glacial acetic acid	5 ml

Prepare just prior to use.

Acetic acid (12%) solution

Glacial acetic acid	24 ml
Distilled water to make up to 200 ml	

Potassium ferrocyanide (5%) solution

Potassium ferrocyanide	5 g
Distilled water	100 ml

Hydrochloric acid (5%) solution

Concentrated hydrochloric acid	5 ml
Distilled water	95 ml

Potassium ferrocyanide-hydrochloric acid

5% potassium ferrocyanide solution	50 ml
5% hydrochloric acid solution	50 ml

Mix just prior to use.

Acid fuchsin stock (1%)

Acid fuchsin	1 g
Distilled water	100 ml

van Gieson working solution

1% acid fuchsin stock	5 ml
Saturated picric acid	95 ml

Method

1. Dewax in xylene and rehydrate through graded ethanols to water.
2. Rinse in 12% acetic acid solution for 1 minute.
3. Cover the sections with the colloidal iron working solution for 1 hour.
4. Rinse in four changes of the 12% acetic acid solution (3 minutes each).

5. Place in the potassium ferrocyanide-hydrochloric acid solution for 20 minutes.
6. Rinse in running tap water for 5 minutes.
7. Rinse briefly in distilled water.
8. Stain with van Gieson working solution for 5 minutes.
9. Dehydrate the specimens in 95% ethanol and absolute ethanol, three changes each. Clear in xylene.
10. Coverslip using an appropriate mounting medium.

Results

Proteoglycans, hyaluronic acid and acidic mucins	bright blue
Collagen	red
Muscle and cytoplasm	yellow

Notes

a. The pH of the colloidal iron working solution is critical. At a pH of 2.0 or higher non-specific staining of structures other than acidic carbohydrate groups will occur.

b. Some protocols may recommend dialysis of the stock colloidal iron solution to remove free acid and unhydrolyzed, ionizable, iron salts. To dialyze the solution, transfer the stock colloidal ion solution in 25 ml portions to 41-mm dialysis tubes suspended in distilled water. Dialyze for 24 hours, changing the water twice during this period. Filter the contents of the dialysis tubes through fine filter paper (Whatman No. 50 or equivalent) to remove any particulate matter (Lillie & Fulmer, 1976).

c. Nuclear fast red may be used as an alternative counterstain for this technique.

d. This technique may be performed in conjunction with the PAS protocol. The colloidal iron technique is performed first and after Step 7 of the protocol the section is subjected to periodic acid oxidation. The remainder of the PAS procedure is performed as described previously.

e. A control slide for each test specimen should be subjected to the potassium ferrocyanide-hydrochloric acid solution only. This is necessary to exclude the possibility that a positive result of the colloidal iron stain is due to the presence of hemosiderin.

f. If the colloidal iron working solution is not available, a solution of 2.73 g of $FeCl_3 \cdot 6H_2O$ in 4.4 ml of distilled water may be used (Lillie & Fulmer, 1976).

High iron diamine

This Spicer technique is useful for the detection of the highly acidic sulfomucins and is selective for carbohydrates carrying a high negative charge density due to ionized sulfate groups. Hyaluronic acid and sialomucins are not demonstrated by this technique (Spicer, 1965; Gad & Sylven, 1969). Combined with the alcian blue protocol, the high iron diamine technique facilitates the differentiation of sulfomucins from sialomucins in tissue sections. With this combination sulfomucins and proteoglycans stain brown to black while sialomucins and hyaluronic acid stain blue. It is well suited to demonstrate the distribution of sialomucins and sulfomucins in the epithelia of the intestines (Spicer, 1965).

Combined high iron diamine and alcian blue technique (modified Spicer, 1965)

Solutions

High iron diamine solution

N,N-dimethyl-*m*-phenylenediamine $(HCl)_2$	120 mg
N,N-dimethyl-*p*-phenylenediamine (HCl)	20 mg

Dissolve the above reagents in 50 ml of distilled water. Pour into a Coplin jar containing 1.4 ml of N.F. 10% ferric chloride ($FeCl_3$, see note c).

Alcian blue solution (in 3% acetic acid) (see page 185).

Method

1. Dewax in xylene and rehydrate through graded ethanols to distilled water.
2. Stain the sections in the high iron diamine solution for 18 hours.
3. Rinse in running tap water for 5 minutes.
4. Stain in the alcian blue solution (pH 2.5) for 30 minutes.
5. Rinse in running tap water for 10 minutes.
6. Dehydrate in graded ethanols and clear with xylene.
7. Coverslip with a miscible mounting medium.

Results

Sulfated mucins and proteoglycans	brown/black
Sialomucins and hyaluronic acid	blue

Notes

a. Diamine salts are toxic; handling should be with great care and kept to a minimum.

b. Nuclear fast red may be used to enhance nuclear contrast.
c. The N.F. 10% ferric chloride solution is equivalent to a 62% w/v solution of $FeCl_3 \cdot 6H_2O$ (Lillie & Fulmer, 1976).

Metachromatic methods

Pearse (1960) defined metachromasia as the staining of tissue or tissue components where the color of the tissue-bound dye complex differs significantly from the color of the original dye complex producing a marked contrast in color. Typically, there is a shift in the absorption of light by the tissue dye complex toward the shorter wavelengths with an inverse shift in color transmission or emission towards the longer wavelengths. Methylene blue, azure A and toluidine blue are small planar cationic dyes which typically stain tissues blue. Metachromatically these dyes stain tissue components purple-red. The use of such dyes to identify charged mucins and proteoglycans is one of the oldest of the histochemical techniques for carbohydrates.

Metachromasia is a specific form of dye aggregation which is characterized by the formation of new intermolecular bonds between adjacent dye molecules (Pearse, 1960). The bonds between the dye molecules only occur when the molecules are brought into close proximity to one another (Sylven, 1954; Bergeron & Singer, 1958), e.g. with acid mucins or proteoglycans, the anionic groups of the carbohydrates act to orient the cationic dye molecules. Simply put, the anionic carbohydrate structure serves as a template to induce the formation of a polymeric dye structure in which the dye molecules bind to one another through hydrogen bonds or van der Waals' forces. Integration of water molecules between adjacent dye molecules is essential for the metachromatic phenomena (Sylven, 1954; Bergeron & Singer, 1958).

A certain pattern of distribution and density of repeating anionic structures is necessary for metachromasia (Pearse, 1960). The highly anionic proteoglycans with alternating sulfate and carboxylate groups meet these criteria and produce metachromatic stains with dyes such as toluidine blue, methylene blue and azure. Generally, the strongly acidic or highly sulfated proteoglycans will produce the strongest and most stable metachromasia (Tonna & Cronkite, 1959; Thompson, 1966).

Post-treatment of sections following staining with metachromasia-producing dyes can have a profound effect on the stability of the metachromasia. Metachromasia may be described as 'alcohol fast' if metachromasia is retained following dehydration, and as 'alcohol labile' if metachromasia is lost in the process (Pearse, 1960). It is recommended that sections are examined first in water prior to their placement in alcohol. These metachromasia-generating techniques have largely been replaced by techniques such as alcian blue and the reader is directed to earlier texts and publications in the literature for more detailed information (Kramer & Windrum, 1955; Bergeron & Singer, 1958; Pearse, 1960; Lillie & Fulmer, 1976).

Azure A technique (Kramer & Windrum, 1955)

Alcoholic azure A solution

Azure A	0.01 g
Ethanol 30%	100 ml

Method

1. Dewax sections in xylene and hydrate through graded ethanols to distilled water.
2. Cover sections with the azure A solution for 10 minutes.
3. Rinse in distilled water.
4. Dehydrate the sections in graded ethanols and clear in xylene.
5. Coverslip using a miscible mounting medium.

Results

Acid mucins and proteoglycans	purple to red
Tissue background	blue

Note

0.1% azure A solution (0.1 g azure A in 100 ml of 30% ethanol) can be used to demonstrate the weakly metachromatic acid carbohydrate-containing molecules.

Lectins

These were initially characterized as proteins isolated from plants which were capable of agglutinating mammalian erythrocytes (Sharon & Lis, 1972). Agglutination occurs because the lectin molecule binds multiple glycoprotein molecules on the cell surface and it may cross-link erythrocytes (Sharon & Lis, 1972; Goldstein & Hayes, 1978). Histochemically, lectins may be defined as plant or animal proteins which bind specific carbohydrate moieties in tissue specimens. Lectin techniques are not routinely performed in histology laboratories so the discussion of lectins is brief and the reader is directed to the following references for a more thorough discussion and review (Sharon & Lis, 1972; Goldstein & Hayes, 1978; Spicer & Schulte, 1992).

Lectins have been isolated from a wide range of animal and plant sources. The more commonly used lectins include concanavalin A from the jack bean, peanut agglutinin, and *Ulex europaeus* (gorse). Lectins principally bind to the terminal carbohydrate molecules of the oligosaccharide or polysaccharide chains of glycoproteins (Goldstein & Poretz, 1986) but the affinity of a lectin may be influenced by the monosaccharide unit adjacent to the terminal unit. Concanavalin A binds terminal mannose moieties, peanut agglutinin binds galactose or galactosamine (Hennigar et al., 1987; Spicer & Schulte, 1992) and *Ulex europaeus* specifically binds L-fucose (Allen et al., 1977).

Lectin molecules can be labeled with fluorochromes such as fluorescein or rhodamine as well as the histochemically detectable enzymes, horseradish peroxidase and alkaline phosphatase (Gonatas & Avrameas, 1973). Labeled in this manner they have been used for a number of purposes in histopathology. *Ulex europaeus* in particular has been used as a valuable marker of normal and neoplastic endothelial cells (Walker, 1985) but its use has now diminished with the emergence of immunohistochemistry and specific antibodies to endothelial markers such as factor VIII, CD31 and CD34.

Immunohistochemistry

Despite the high sensitivity and specificity of immunohistochemistry these techniques are rarely used for the routine evaluation of tissue specimens for glycogen or the proteoglycans and the special stains described in this chapter remain the standard means for the evaluation of these substances. Immunohistochemistry has however become an invaluable tool for the detection of a variety of specific mucins and mucin-like molecules which are markers of the neoplastic process. Examples of these types of molecule are epithelial membrane antigen (EMA) and the tumor-associated glycoprotein (TAG-72) (see Chapter 19).

Enzymatic digestion techniques

Various enzymatic digestion techniques have been applied to increase or verify the specificity of carbohydrate staining. For example, the amylase or diastase techniques for glycogen digestion are commonly utilized in laboratories to enhance the specificity of the PAS technique. The neuraminidase and hyaluronidase techniques described below however are more likely to be performed in research laboratories with an interest in a specific area of glycobiology.

Diastase digestion

The PAS technique is unique among the methods described in this chapter in that it detects a varied number of mucosubstances, e.g. glycogen, mucins, and glycoproteins. The distinction between mucins and glycogen can be problematic when using the PAS technique and the inclusion of a glycogen digestion step is necessary when the diagnosis requires the correct identification between these mucosubstances. α-Amylase may be used in this situation as it catalyzes the hydrolysis of the glycosidic bonds of glycogen and the breakdown of the large glycogen molecules to the water-soluble disaccharide, maltose (Bernfeld, 1951). The net result is the removal of glycogen from the tissue section prior to the PAS technique. Malt diastase contains both α- and β-amylases and is frequently used for this purpose (Lillie et al., 1947). Human saliva is an effective means of digesting glycogen but its use is discouraged for safety reasons and the lack of standardization of preparations. Duplicate slides are required when a glycogen digestion procedure is used.

Diastase digestion (Lillie & Fulmer, 1976)

Solutions

Phosphate buffer

Monobasic sodium phosphate	1.97 g
Dibasic sodium phosphate	0.28 g
Distilled water	1000 ml

This solution may be kept in the refrigerator for several months.

Diastase solution

Malt diastase	0.1 g
Phosphate buffer	100 ml

Method

1. Dewax two serial sections in xylene and rehydrate through graded ethanols to water.
2. Place one slide in the diastase solution for 1 hour at 37°C. The other slide is an untreated control and may remain in water for 1 hour.
3. Wash both slides in running tap water for 5–10 minutes.
4. Proceed with the PAS technique.

Results (with PAS procedure)

Glycogen should demonstrate bright red/magenta staining in the untreated slide. Glycogen staining should be absent in the diastase treated slide.

Notes

- **a.** A known positive control should be included to verify the potency of the enzyme.
- **b.** Commercial batches of diastase or amylase may vary widely in activity and purity. Contaminating enzymes may digest material other than glycogen.
- **c.** Alpha-amylase may be used instead of malt diastase.

Sialidase (neuraminidase)

This enzyme is isolated from the bacterium *Vibrio cholerae* (Kiernan, 1999) and specifically cleaves the terminal sialic acid moieties from sialomucins and glycoproteins (Drzeniek, 1973). The loss of PAS or alcian blue staining following sialidase treatment is clearly indicative of the presence of sialic acid in tissue specimens. If the combined alcian blue-PAS protocol is performed following sialidase treatment, sialomucins which normally stain blue with alcian blue, stain red with PAS. Removal of the alcian blue reactive anionic carboxylate group containing sugars from these mucins renders the mucin reactive with PAS.

By contrast if staining is lost following treatment, a lack of effect of sialidase is difficult to interpret. It may indicate a lack of sialic acid in the specimen or the possibility of the sialidase-resistant *O*-acetylated sialic acids. These resistant sialic acids can be converted to a sialidase labile form by the deacetylation procedure (Ravetto, 1968). This technique uses an alkaline (ammonia) alcohol solution to remove the *O*-acetyl groups from the sialic acid. Subsequent treatment with sialidase cleaves the previously enzyme-resistant sialic acids. A comparison of alcian blue (pH 2.5) staining in sections subjected to the deacetylation-sialidase combination to the staining of sections exposed only to sialidase will reveal the presence of *O*-acetyl group-containing sialic acids.

Sialidase digestion (Bancroft & Cook, 1994)

Sialidase solution

One unit/ml sialidase ex. *V. cholerae* diluted 1 in 5 with 0.2 M acetate buffer, pH 5.5. Add 1% calcium chloride w/v. The activity of the diluted enzyme will persist for a few weeks if stored at 4°C.

Method

1. Dewax two sections from a positive control and two sections from each test specimen and bring sections to water.
2. Rinse the sections with buffer and treat one positive control section and one section from each test specimen with the sialidase solution for 16–24 hours at 37°C. The remaining slides (one positive control section and one section per test specimen) are incubated in buffer (37°C) alone for the same period of time.
3. Rinse in running tap water for 5 minutes.
4. Proceed to alcian blue or the alcian blue-PAS technique.

Results

Sialidase-labile sialic acids will stain bright blue in the untreated section. This staining is lost following sialidase treatment. Alcian blue-PAS mucins containing sialidase-labile sialic acids will stain bright blue while neutral mucins stain red to magenta in the untreated sections. Following treatment, mucins containing

sialidase-labile sialic acids will stain red to magenta secondary to the PAS stain (Spicer & Warren, 1959).

Notes

a. Sialic acids containing an *O*-acetyl group are usually resistant to sialidase.
b. A known positive control should be included to verify the potency of the enzyme.

Hyaluronidase

This enzyme cleaves the glycosidic linkages of hyaluronic acid and, depending upon the source of the enzyme, glycosidic linkages in other glycosaminoglycans. The most commonly used hyaluronidase is isolated from an extract of bull testes. This enzyme is not specific as it removes hyaluronic acid but also breaks the glycosidic linkages of the chondroitin sulfates (Meyer & Rapport, 1952). It may be used as a pretreatment of specimens prior to staining with alcian blue or colloidal iron. The loss of staining when compared to a non-treated duplicate section is indicative of the presence of hyaluronic acid or the chondroitin sulfates; conversely, if the pretreatment has no effect, the presence of hyaluronic acid is unlikely. Despite the lack of specificity, a negative response to bovine testicular hyaluronidase can be used to rule out the possible presence of hyaluronic acid.

A form of hyaluronidase isolated from several bacterial species also has been used for the identification of hyaluronic acid (Meyer & Rapport, 1952). These enzymes are more selective than bovine testicular hyaluronidase and have been shown to act specifically on hyaluronic acid.

Hyaluronidase digestion (Gaffney, 1992)

Solutions

Phosphate buffer solution

Sodium chloride	8 g
Monobasic sodium phosphate	2 g
Dibasic sodium phosphate	0.3 g
Distilled water	1000 ml

Hyaluronidase solution

Bovine testicular hyaluronidase	50 mg
Phosphate buffer solution	100 ml

Method

1. Dewax two sections from a positive control and two sections from each test specimen. Bring sections to distilled water.
2. Incubate one positive control section and one section from each test specimen with the hyaluronidase solution for 3 hours at 37°C. The remaining slides (one positive control and one section per test specimen) should be treated with buffer alone for 3 hours at 37°C.
3. Wash all slides in running tap water for 5 minutes.
4. Perform staining technique, e.g. alcian blue.

Results with alcian blue pH 2.5

Connective tissue proteoglycans containing chondroitin sulfate and/or hyaluronic acid stain bright blue in sections not treated with hyaluronidase. This staining is lost following hyaluronidase treatment.

Note

A known positive control is necessary to verify the potency of the enzyme. A good control is umbilical cord which contains Wharton's jelly, a substance rich in hyaluronic acid.

Chemical modification and blocking techniques

There are a number of techniques which have been used to block the reactive groups of carbohydrates. Blocking these groups, e.g. hydroxyls, carboxyls and sulfate esters prevents the effect of the reagents used in subsequent histochemical techniques. Blocking techniques are used rarely in today's histology laboratory but are particularly useful for determining specific types of carbohydrate present in a tissue specimen.

Methylation

There are a number of variations of this technique which have been used to identify carboxyl or sulfate acid groups in mucins, all of which are based on the treatment of the specimens with an acidified methanolic solution. In the 'mild' technique, the sections are exposed to the solution for a relatively short period, 4 hours at 37°C (Spicer, 1960). Under these conditions, the carboxylate groups of the mucins are converted to methyl esters. The loss of alcian blue pH 2.5 reactivity within a

specimen following this procedure is indicative of a predominance of carboxylated carbohydrates in the tissue. Conversely, any staining following this procedure is due to sulfate-containing carbohydrates.

Treatment of specimens with an acidified methanolic solution for 5 hours or more at 60°C converts carboxylate groups to methyl esters and also removes or hydrolyzes *O*-sulfate and *N*-sulfate groups in mucins and proteoglycans. Sections treated in this manner should demonstrate a total loss of alcian blue reactivity. This technique is of little value if used alone and is frequently performed with the saponification technique.

Mild methylation technique (Spicer, 1960)

Acid methanol solution

Concentrated hydrochloric acid	0.8 ml
Methanol	99.2 ml

Method

1. Dewax two sections from a positive control and two sections from each test specimen. Bring sections to distilled water.
2. Place one positive control section and one section from each test specimen in preheated (37°C) acid methanol solution for 4 hours. The remaining slides (one positive control and one slide per test specimen) should be placed in distilled water at 37°C for 4 hours.
3. Wash in running tap water for 5 minutes.
4. Perform alcian blue (pH 2.5) stain.

Results with alcian blue pH 2.5

Sulfated mucins and proteoglycans, sialomucins and hyaluronic acid will stain bright blue in the untreated specimen. A diminution of staining following treatment with the acid methanol reflects a loss of stainable sialomucins and/or hyaluronic acid. Any staining which remains following treatment is due to sulfomucins and/or sulfate proteoglycans.

Note

Treatment beyond 4 hours may result in the hydrolysis of sulfate groups. Positive control slides are necessary to verify the effectiveness of the methylation procedure.

Saponification

The alkaline alcoholic solution used in this technique cleaves the linkage of the *O*-acetyl groups within the enzyme-resistant sialic acids. As with the deacetylation procedure described above, this technique renders the *O*-acetylated sialic acids sensitive to sialidase. The removal of the *O*-acetyl group and the restoration of the hydroxyl groups on the C7–C9 side chain of sialic acid also restore the PAS reactivity of the sialic acids (Culling et al., 1974).

This technique is also used to reverse the effects of methylation. Saponification cleaves the bonds of the methyl esters formed during methylation and restores the carboxylate groups (Spicer & Lillie, 1959). Following the methylation protocol, saponification will restore the alcian blue staining of the carboxylated carbohydrates. Sulfate esters which are lost due to the more aggressive methylation protocol are not restored by saponification. The restoration of alcian blue staining following saponification is due to the presence of sialomucins or hyaluronic acid.

Combined methylation-saponification technique (Spicer & Lillie, 1959)

Solutions

Acid methanol solution

See above.

Saponification solution

Potassium hydroxide	1 g
Ethanol	70 ml
Distilled water	30 ml

Method

1. Dewax three positive control sections and three sections from each test specimen. Bring sections to water.
2. Place two positive controls and two test sections in the acid methanol solution at 60°C for 5 hours. The additional positive control and test specimen should be placed in 60°C distilled water for 5 hours.
3. Wash all sections in running tap water for 5 minutes.
4. Place one each of the positive control sections and test sections which were treated with acid methanol (Step 2) in the saponification solution for 30 minutes at room temperature. Place all other sections in 70% ethanol at room temperature for 30 minutes.

5. Wash for 5 minutes.
6. Stain with alcian blue pH 2.5.

Results

a. Sections without methylation or saponification: sulfated and carboxylated mucins, proteoglycans and hyaluronic acid will stain bright blue.
b. Sections treated with acid methanol but not subjected to saponification demonstrate little or no alcian blue staining.
c. Sections treated with acid methanol and subjected to saponification: the carboxylated mucins and hyaluronic acid should demonstrate bright blue staining with alcian blue. Any loss of staining when compared to the sections which were not treated with acid methanol or subjected to saponification (result a) is due to the presence of sulfated mucins and/or sulfated proteoglycans.

Notes

a. Silanized slides should be used.
b. Celloidinization of slides has been recommended to reduce tissue loss during the saponification process.

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14 Pigments and minerals

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Introduction

In biology, pigments are defined as substances occurring in living matter which absorb visible light (electromagnetic energy within a narrow band which lies approximately between 400 and 800 nm). The various pigments may greatly differ in origin, chemical constitution and biological significance. They can be either organic or inorganic compounds which remain insoluble in most solvents. Minerals are naturally occurring homogeneous, inorganic substances having a definite chemical composition and characteristic crystalline structure, color and hardness. In biology they are necessary for growth and development.

Pigments can be classified under the following headings

1. Endogenous pigments

These substances are produced either within tissues and serve a physiological function, or are by-products of normal metabolic processes. They can be further subdivided into:

- Hematogenous (blood-derived) pigments
- Non-hematogenous pigments
- Endogenous minerals.

2. Artifact pigments

These are deposits of artifactually produced material caused by the interactions between certain tissue components and some chemical substances, such as the fixative formalin. Formalin and malaria pigments are sometimes classified as a subdivision of endogenous pigments.

3. Exogenous pigments and minerals

These substances gain access to the body accidentally through a variety of methods e.g. carbon anthracotic pigment, which is seen in lung and lymph nodes. These pigments and minerals serve no physiological function. Entry is gained either by inhalation into the lungs or by implantation e.g. tattoos, into the skin. Most exogenous pigments are minerals, few of which are pigmented.

The above classification, although not scientifically precise, is used as a convenient aid in the identification of pigments. It is important to bear in mind that the same pigment may present itself in tissue sections in a variety of ways. Iron, for example, may be present as an endogenous pigment in liver sections in an iron overload condition, and as an exogenous pigment in the case of a shrapnel wound/prosthetic implantation. It is advisable to note a pigment's morphology, tissue site and relevant clinical data before carrying out the various stains and histochemical reactions available in order that a pigment can be identified.

Endogenous pigments

Hematogenous

This group contains the following blood-derived pigments:

- Hemosiderins
- Hemoglobin
- Bile pigments
- Porphyrins.

Hemosiderins

These pigments are seen as yellow to brown granules and normally appear intracellularly. They contain iron in the form of ferric hydroxide which is bound to a protein framework and is unmasked by various

chemicals. Iron is a vital component of the human body as it is an essential constituent of the oxygen-carrying hemoglobin found in red blood cells, where 60% of the body's total iron content resides. It also occurs in myoglobin and certain enzymes, such as cytochrome oxidase and the peroxidases.

Normal and abnormal iron metabolism

Dietary iron is absorbed in the small intestine and attached to a protein molecule for transfer to the sites in the body where it is to be utilized or stored. Approximately 30% is stored within the reticuloendothelial system, especially the bone marrow. The bone marrow is the main site of iron utilization in the body and where it is incorporated into the hemoglobin molecule during red cell formation. The normal breakdown of worn-out red cells results in the release of iron which is recirculated back into the various areas of iron storage for further utilization. Under normal conditions, this efficient system of recycling usually means that iron deficiency rarely occurs. There is a minimal loss of iron by the way of epithelial desquamation, hair loss and sweating. The main reason for loss of iron from the body is hemorrhage in the form of either chronic bleeding (e.g. a peptic ulcer or bowel neoplasia), or in the female by menstruation, with approximately 25% of females being iron deficient. The small intestine normally only absorbs sufficient iron from the excess iron in the diet to counteract any losses, but in excessive blood loss the dietary content may be relatively inadequate for the need, and even though the absorption mechanism is working at full efficiency, a state of clinical iron deficiency occurs. In iron deficiency, the iron stores in the bone marrow become depleted, insufficient hemoglobin is produced because of the lack of iron, and anemia develops in which the red cells contain diminished amounts of hemoglobin. The iron deficiency is characteristically demonstrated by the absence of stainable iron in the bone marrow.

There is no active method of iron excretion from the body. Iron excess is a much less common condition; under normal conditions the intestine will not absorb iron from the diet when there is already a surplus within the body. However, this controlling mechanism may be bypassed when iron is given therapeutically, such as in the form of either iron injections or a blood transfusion. If excess iron is given this way, the iron stores may become overloaded, and excessive amounts of hemosiderin may be deposited in the organs with a prominent reticuloendothelial component e.g. spleen, bone marrow or liver. This condition is called *hemosiderosis*. A rarer cause of iron overload is the genetic disease *hemochromatosis*, in which the controlling mechanism at the small intestine absorption stage becomes impaired, and the iron is absorbed indiscriminately in amounts irrelevant to the state of the body's iron stores. In this disorder, large quantities of hemosiderin are deposited in many of the organs, often interfering with those organs' structure and function.

Demonstration of hemosiderin and iron

In unfixed tissue, hemosiderin is insoluble in alkalis but freely soluble in strong acid solutions. After fixation in formalin, it is slowly soluble in dilute acids, especially oxalic acid. Fixatives which contain acids but no formalin can remove hemosiderin or alter it in such a way that reactions for iron are negative. Certain types of iron found in tissues are not demonstrable using traditional techniques. This is because the iron is tightly bound within a protein complex. Both hemoglobin and myoglobin are examples of such protein complexes and, if treated with hydrogen peroxide (100 vol), the iron is released and can then be demonstrated using Perls' Prussian blue reaction (Fig. 14.1). A similar result is obtained if the acid ferrocyanide solution is heated to 60°C in a water bath, oven or microwave oven. However, the use of heat will sometimes cause a fine, diffuse, blue precipitate to form on both the tissue section and slide. This precipitate will not occur when the slides are stained at room temperature. Metallic iron deposits, or inert iron oxide seen in tissues because of industrial exposure, are not positive when treated with acid ferrocyanide solutions. As a consequence of the tissue response, various mechanisms release some of the iron in a demonstrable form, and such deposits are almost invariably surrounded by hemosiderin. In almost all the instances where demonstrable iron appears in tissues, it does so in the form of a ferric salt. On those rare occasions

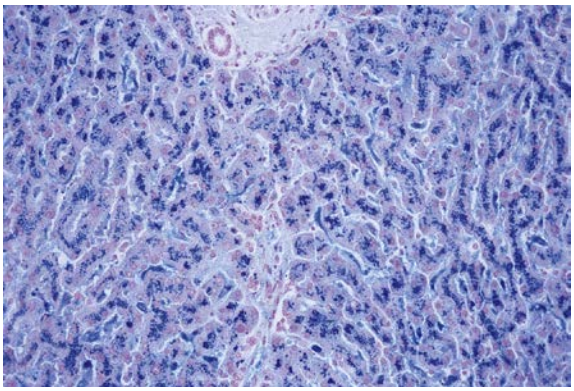


Fig. 14.1 A section of liver from a patient with hemochromatosis stained for ferric iron with Perls' method. Ferric iron is stained blue.

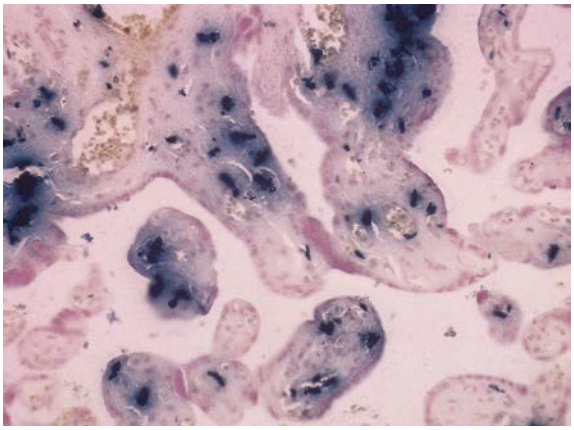


Fig. 14.2 A section of placenta treated with ferrous sulfate and stained with Lillie's method for ferrous iron. Ferrous iron is stained dark Turnbull blue.

when iron appears in its reduced state as the ferrous salt, then Lillie's (1965) method may be used to achieve the Turnbull's blue reaction to visualize its presence in tissue sections (Fig. 14.2).

An interesting and sometimes useful modification of a serum iron technique was introduced by Hukill and Putt (1962) to demonstrate both ferrous and ferric iron in tissue sections. This method was claimed to be a more sensitive demonstration for the detection of both ferric and ferrous salts, but has not succeeded in replacing the more traditional method for the demonstration of iron. The method uses bathophenanthroline and the resultant color of any iron present in tissues is bright red.

Perls' Prussian blue reaction for ferric iron (Perls, 1867)

This method is considered to be the first classical histochemical reaction. Treatment with an acid ferrocyanide solution will result in the unmasking of ferric iron in the form of the hydroxide, $Fe(OH)_3$. The ferric iron then reacts with a dilute potassium ferrocyanide solution to produce an insoluble blue compound, ferric ferrocyanide (Prussian blue).

Fixation

Avoid the use of acid fixatives. Chromates will also interfere with the preservation of iron.

Sections

Works well on all types of section, including resin.

Acid ferrocyanide solution

1% aqueous potassium ferrocyanide	20 ml
2% aqueous hydrochloric acid	20 ml

Preferably freshly prepared, mix just before use.

Method

1. Take a test and control section to water.
2. Treat sections with the freshly prepared acid ferrocyanide solution for 10–30 minutes (see Note a below).
3. Wash well in distilled water.
4. Lightly stain the nuclei with 0.5% aqueous neutral red or 0.1% nuclear fast red.
5. Wash rapidly in distilled water.
6. Dehydrate, clear and mount in synthetic resin.

Results

Ferric iron	blue
Nuclei	red

Notes

a. Depending on the amount of ferric iron present, it may be necessary to vary the staining times.

b. Some laboratories keep the two stock solutions made up separately and stored in the refrigerator. The two solutions must not be stored for prolonged periods; this precaution will ensure that the solutions retain their viability.

c. It is essential that a positive control is used with all test sections. The choice of material which is most suitable as a control specimen is important. A useful control would be postmortem lung tissue which contains a reasonable number of iron-positive macrophages (heart failure cells). Freshly formed deposits of iron may be dissolved in the hydrochloric acid.

Lillie's method for ferric and ferrous iron
(Lillie & Geer, 1965)

Fixation

Avoid the use of acid fixatives. Chromates will also interfere with the preservation of iron.

Sections

Paraffin wax, frozen and resin.

Method

1. Take test and control sections to distilled water.
2. Dissolve 400 mg of potassium ferrocyanide in 40 ml of 0.5% hydrochloric acid when testing for ferric iron. For testing ferrous iron, substitute 400 mg of potassium ferricyanide. Prepare just before use. Expose sections for 30 minutes.
3. Wash well in distilled water.
4. Stain nuclei with 0.1% aqueous nuclear fast red for 5 minutes.
5. Rinse in distilled water.
6. Dehydrate, clear, and mount in synthetic resin.

Results

Ferric iron	dark Prussian blue
Ferrous iron	dark Turnbull's blue
Nuclei	red

Hukill and Putt's method for ferrous and ferric iron
(Hukill & Putt, 1962)

Fixation

Not critical but avoid prolonged exposure in acid fixatives.

Sections

All types of tissue section may be used including resin.

Solution

Bathophenanthroline (4, 7-diphenyl-1, 10-phenanthroline)	100 mg
3% aqueous acetic acid	100 ml

Place together in oven at 60°C for 24 hours, agitating at regular intervals. Cool to room temperature and filter. This solution is stable for about 4 weeks. Before use, add thioglycolic acid to a concentration of 0.5% (this should be replenished each time before use as it rapidly undergoes oxidation on exposure to air).

Method

1. Take test and control sections to distilled water.
2. Stain sections in bathophenanthroline solution for 2 hours at room temperature.
3. Rinse well in distilled water.
4. Counterstain in 0.5% aqueous methylene blue for 2 minutes.
5. Rinse well in distilled water.
6. Stand slides on end until completely dry.
7. Dip slides in xylene and mount in synthetic resin.

Results

Ferrous iron	red
Nuclei	blue

Notes

a. It is important that the bathophenanthroline is completely dissolved prior to use.
b. Dehydration with alcohol will remove the resultant red coloration.

Hemoglobin

Hemoglobin is a basic conjugated protein which is responsible for the transportation of oxygen and carbon dioxide within the bloodstream. It is composed of a colorless protein, globin, and a red pigmented component, heme. Four molecules of heme are attached to each molecule of globin.

Heme is composed of protoporphyrin, a substance built up from pyrrole rings and combined with ferrous iron. Histochemical demonstration of the ferrous iron is possible only if the close binding in the heme molecules is cleaved. This can be achieved by treatment with hydrogen peroxide, but this has no practical use. As hemoglobin appears normally within red blood cells its histological demonstration is not usually necessary. The need to demonstrate the pigment may arise in certain pathological conditions e.g. casts in the lumen of renal tubules in cases of hemoglobinuria or active glomerulonephritis.

Demonstration of hemoglobin

Two types of demonstration method can be used to stain hemoglobin in tissue sections. The first demonstrates the enzyme, hemoglobin peroxidase, which is reasonably stable and withstands

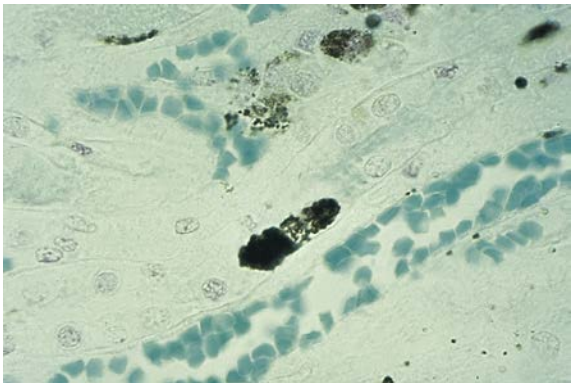


Fig. 14.3 A section of kidney from a patient with hemoglobinuria stained for hemoglobin with leuco patent blue V. Hemoglobin is stained blue.

short fixation and paraffin processing. This peroxidase activity was originally demonstrated by the benzidine-nitroprusside methods, but because of the carcinogenicity of benzidine these methods are not recommended and are no longer used. Lison (1938) introduced the patent blue method which was later modified by Dunn and Thompson (1946) (Fig. 14.3). Tinctorial methods have also been used for the demonstration of hemoglobin; the amido black technique (Puchtler & Sweat, 1962) and the kiton red-almond green technique (Lendrum, 1949) are worth noting.

Leuco patent blue V method for hemoglobin
(Dunn & Thompson, 1946)

Fixation

Formalin is best; poor preservation with Heidenhain's Susa has been noted by Drury and Wallington (1980).

Solutions

Stock solution

1% aqueous patent blue V (CI 42045)	25 ml
Powdered zinc	2.5 g
Glacial acetic acid	0.5 ml

Mix well on a magnetic stirrer. The solution will become pale green-blue. Filter and store in a refrigerator at 3–6°C. The solution is stable for about 1 week.

Working solution

Stock solution	10 ml
Glacial acetic acid	2 ml
3% hydrogen peroxide	1 ml

Prepare immediately before use.

Method

1. Take test and control sections to distilled water.
2. Stain in patent blue solution for 5 minutes at room temperature.
3. Rinse in distilled water.
4. Lightly counterstain in 0.5% aqueous neutral red or 0.1% aqueous nuclear fast red for 1 minute.
5. Rinse in distilled water.
6. Dehydrate, clear, and mount in synthetic resin.

Results

Hemoglobin peroxidase (red blood cells and neutrophils)	dark blue
Nuclei	red

Notes

a. Experience has shown that the hemoglobin demonstrated by this method tends to be more of a green-blue color.
b. In some instances, if the staining solution is left on the section for too long, it may start to decolor a positive reaction.
c. Fixation in excess of 36 hours may give rise to unreliable results.
d. This method demonstrates peroxidase activity, including the peroxidases in other blood cells, particularly in the lysosomes of polymorphonuclear leukocytes and their precursors. Tissue peroxidases are also demonstrated.

Bile pigments

Red blood cells are broken down in the reticuloendothelial system when they have reached the end of their useful life, usually after 120 days. Hemoglobin is released after the red cell membrane has been ruptured. The protein, globin and iron components are released for recycling within the body after the hemoglobin has been broken down. After the heme portion has been split from the globin, the tetrapyrrole ring of the heme molecule is cleaved and opened out into a chain composed

of four linked pyrrole groups. With the opening out of the tetrapyrrole ring, the iron component is removed to be stored in those tissues which specialize in iron storage. This iron component is now free to be incorporated into the hemoglobin molecule during red cell formation. The opened tetrapyrrole ring, which has had its iron component removed, is known as biliverdin. This residue is formed in the phagocytic cells which populate the reticuloendothelial system, particularly the bone marrow and spleen. Biliverdin is transported to the liver, where it is reduced to form bilirubin. In this form, the bilirubin is insoluble in water, but after conjugation with glucuronic acid it forms a water-soluble compound, bilirubin-glucuronide. This process takes place in liver hepatocytes due to the activity of the enzyme glucuronyl transferase. The conjugated bilirubin passes from the hepatocytes into the bile canaliculi, and then via the hepatic ducts into the gallbladder which acts as a reservoir. The bilirubin passes along the common bile duct to be released into the duodenum via the ampulla of Vater.

The term *bile pigments* used by many authors when discussing the various (generic) staining techniques can be applied to all bile pigments. In using this terminology, it is implied that all bile pigments react in an identical manner, but this is not the case. Contained within the group 'bile pigments' are both conjugated and unconjugated bilirubin, biliverdin, and hematoidin, all of which are chemically distinct and show different physical properties, particularly with regard to their solubility in water and alcohol. Microscopic examination of any liver section which contains 'bile pigments' will almost certainly reveal a mixture of biliverdin and both conjugated and unconjugated bilirubin. This is particularly likely when the liver contains an excess of bile pigments, either through bile duct obstruction, e.g. due to a stone or tumor, an abnormality of biliverdin-bilirubin metabolism in the rare congenital enzyme disorders, or where there is extensive liver cell death or degeneration. The non-specific term *bile* will be used to include biliverdin and both conjugated and unconjugated bilirubin in the following text.

In a hematoxylin and eosin (H&E) stained section of liver, bile, if present, is most commonly seen in the hepatocytes in the early stages as small yellow-brown globules and then subsequently within the bile canaliculi as larger, smooth, round-ended rods or globules commonly referred to as *bile thrombi*. The latter, if present, in liver sections is a histopathological indication that the patient has obstructive jaundice due to a blockage in the normal flow of bile from the liver into the gallbladder and subsequently into the bowel, probably because of gallstones or a carcinoma of the head of pancreas. Masses of bile in the canaliculi of liver sections are easily distinguished microscopically because of their characteristic morphology and their situation. Bile in hepatocytes must be distinguished from the lipofuscins which are also commonly seen within these cells and can appear as small yellow-brown globules. The need to distinguish between bile and lipofuscin in hepatocytes is particularly important in liver biopsies taken from liver transplant patients where sepsis is suspected. Bile is difficult to identify in the sections of normal liver. It is important to note that both bile and lipofuscin can be positive with Schmorl's ferric ferricyanide reduction test (Golodetz & Unna, 1909). Bile is also seen in H&E-stained sections in the gallbladder where it can appear as amorphous, yellow-brown masses adherent to the mucosa or included as yellow-brown globules within the epithelial-lined Aschoff-Rokitansky sinuses in the gallbladder. Bile is also present, together with cholesterol, in gallstones.

Virchow (1847) first described extracellular yellow-brown crystals and amorphous masses within old hemorrhagic areas, which he called hematoidin. Pearse (1985) reviewed the histochemistry of bile pigments. Microscopically, *hematoidin* frequently appears as a bright yellow pigment in old splenic infarcts, where it contrasts well against the pale gray of the infarcted tissue. Hematoidin can also be found in old hemorrhagic areas in the brain. Bearing in mind the differences discussed above, it is almost certain that hematoidin is related to both bilirubin and biliverdin, even though it differs from them both morphologically

and chemically. It is thought that heme has undergone a chemical change within these areas which has led to it being trapped, thus preventing it from being transported to the liver to be processed into bilirubin.

Demonstration of bile pigments and hematoidin

The need to identify bile pigments arises mainly in the histological examination of the liver, where distinguishing bile pigment from lipofuscin may be of significant importance. Both appear yellow-brown in H&E-stained paraffin wax sections, and it is worth remembering that the green color of biliverdin is often masked by eosin. In such cases, unstained paraffin wax or frozen sections, lightly counterstained with a suitable hematoxylin (e.g. Mayer's), will prove of value. Bile pigments are not autofluorescent and fail to rotate the plane of polarized light (monorefringent), whereas lipofuscin is autofluorescent. The most commonly used routine method for the demonstration of bile pigments is the modified Fouchet technique (Hall, 1960), in which the pigment is converted to the green color of biliverdin and blue cholecyanin by the oxidative action of the ferric chloride in the presence of trichloroacetic acid (Fig. 14.4). The Fouchet technique is quick and simple to carry out, and when counterstained with van Gieson's solution the green color is accentuated.

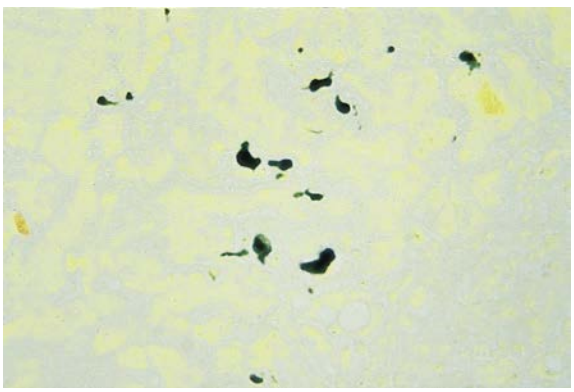


Fig. 14.4 Bile in a section of liver stained with Hall's method for bilirubin. Bilirubin is stained emerald green.

Modified Fouchet's technique for liver bile pigments (Hall, 1960)

Fixation

Any fixative appears suitable.

Sections

Any.

Solutions

Fouchet's solution

25% aqueous trichloroacetic acid	36 ml
10% aqueous ferric chloride	4 ml

Freshly prepared before use.

van Gieson stain

Dissolve 100 mg of acid fuchsin (CI 42685) in 100 ml of saturated aqueous picric acid (see Note c below).

Method

1. Take test and control sections to distilled water.
2. Treat with the freshly prepared Fouchet's solution for 10 minutes.
3. Wash well in running tap water for 1 minute.
4. Rinse in distilled water.
5. Counterstain with van Gieson solution for 2 minutes.
6. Dehydrate, clear and mount in synthetic resin.

Results

Bile pigments	emerald to blue-green
Muscle	yellow
Collagen	red

Notes

a. Two control sections are stained with the test section, one stained with Fouchet's reagent and van Gieson, and the other with Fouchet's reagent alone.

b. Although the solutions used in Fouchet's reagent have a reasonable shelf life, experience has shown that a freshly prepared solution gives a more reliable result.

c. Bile which may be present in situations outside the liver such as that seen in the Aschoff-Rokitansky sinuses or in hemorrhagic and infarcted areas is likely to show no color change with this method. This type of pigment can be shown using the Gmelin (see below) or the Stein technique. Sirius Red F3B (CI 35780) may be substituted for acid fuchsin. Bile is a reducing substance and therefore will stain with the Masson-Fontana and Schmorl techniques.

Gmelin technique (Tiedermann & Gmelin, 1826)

This technique is the only method which shows an identical result with liver bile, gallbladder bile and hematoidin. The method tends to be messy, capricious, and gives impermanent results. Deparaffinized sections of tissue containing bile pigments are treated with nitric acid, and a changing color spectrum is produced. It can be unreliable and so it is advisable to repeat the test at least three times before a negative result is acceptable. A popular modification of this technique is that of Lillie and Pizzolato (1967), in which bromine in carbon tetrachloride is used as an oxidant.

Sections

Paraffin wax embedded.

Method

1. Take sections to distilled water and mount in distilled water.
2. Place mounted section under the microscope using an objective with reasonable working distance.
3. Place 2–3 drops of concentrated nitric acid to one side of the coverglass and draw under the coverglass by means of a piece of blotting paper on the opposite side.
4. Remove excess solution and observe pigment for color changes.

Results

Bile pigments will gradually produce the following spectrum of color change: yellow-green-blue-purple-red.

Notes

a. This method is impermanent, thus preventing storage of sections.
b. The reaction can occur rapidly, but by using a 50–70% solution of nitric acid it can be slowed down.
c. Sulfuric acid can also be used in this method.

Oxidation methods aim to demonstrate bilirubin by converting it to green biliverdin. In practice they fail to produce the bright blue-green color seen in the more popular Fouchet technique and tend to be a dull olive green color. These oxidation methods are of little value in routine surgical pathology and are rarely used. Another group of methods which have been used to demonstrate bile pigments are the diazo methods which are based on a well-known technique previously used in chemical pathology, namely the van den Burgh test for bilirubin in blood. The method is based on the reaction between bilirubin and diazotized sulfanilic acid. Raia (1965, 1967) modified the method for use on cryostat sections but the reagents are complex to make up and section loss may be high, therefore, its use is limited.

Porphyrin pigments

These substances normally occur in tissues in only small amounts. They are considered to be precursors of the heme portion of hemoglobin. The porphyrias are rare pathological conditions which are disorders of the biosynthesis of porphyrins and heme.

In erythropoietic protoporphyria, porphyrin pigment can be seen as focal deposits in liver sections. The pigment appears as a dense, dark brown pigment and in fresh frozen sections exhibits a brilliant red fluorescence which rapidly fades with exposure to ultraviolet light. The pigment, when seen in paraffin wax sections and viewed using polarized light, appears bright red in color with a centrally located, dark Maltese cross.

Non-hematogenous endogenous pigments

This group contains the following:

- Melanins
- Lipofuscins
- Chromaffin
- Pseudomelanosis (melanosis coli)
- Dubin-Johnson pigment
- Ceroid-type lipofuscins
- Hamazaki-Weisenberg bodies.

Melanins

Melanins are a group of pigments whose color varies from light brown to black. The pigment is normally found in the skin, eye, substantia nigra of the brain and hair follicles (a fuller account of these sites is given later). Under pathological conditions, it is found in benign nevus cell tumors and malignant melanomas. The chemical structure of the melanins is complex and varies from one type to another. Melanin production is not fully understood but the

generally accepted view is that melanins are produced from tyrosine by the action of an enzyme tyrosinase (DOPA oxidase). This enzyme acts on the tyrosine slowly to produce the substance known as DOPA (dihydroxyphenylalanine) which is subsequently rapidly acted upon by the same enzyme to produce an intermediate pigment which then polymerizes to produce melanin. The later stages of melanogenesis remain largely speculative, and it is beyond the scope of this chapter to evaluate the many studies relating to melanin biosynthesis which have been carried out recently. Pearse (1985) gives a more detailed account of melanin production.

The melanins are bound to proteins, and these complexes are localized in the cytoplasm of cells within so-called 'melanin granules'. Ghadially (1982) described these granules as the end stage of the development of the melanosome as seen at ultrastructural level.

There are four recognized stages of melanosome maturation:

1. Tyrosine is synthesized in the Golgi lamellae and pinched off into vesicles with no melanin present.
2. The characteristic lattice-like appearance becomes evident at this stage.
3. Melanin deposition is first observed.
4. The fully mature granule has its structure obscured by melanin pigment.

Ultrastructurally the lamellar structures become increasingly difficult to see following melanin deposition. By the time the melanosome reaches stage 4 the lamellae structures are completely obscured (Fig. 14.5).

The enzyme tyrosinase cannot be demonstrated in the mature granule.

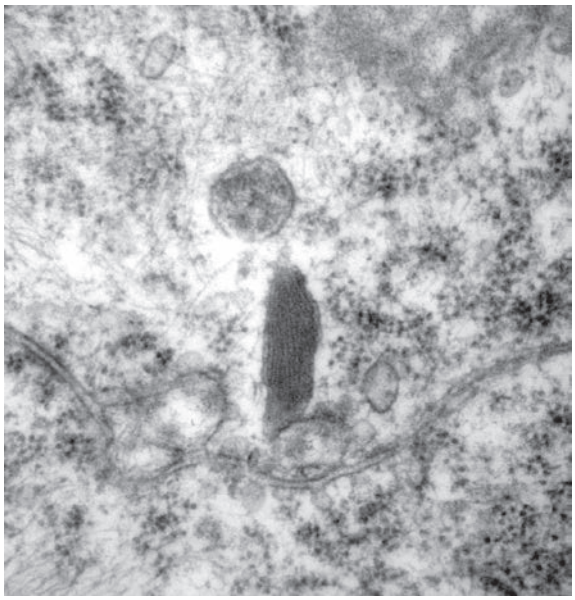


Fig. 14.5 Transmission electron micrograph of a stage 3 melanosome, demonstrating the characteristic internal lamellae structure. (Courtesy of Ms Tracey Deharo.)

The most common sites where melanin can be found are:

1. *Skin*, where it is produced by cells called melanocytes which are usually scattered within the basal layer of the epidermis. In certain inflammatory skin diseases melanin may also be found in phagocytic cells ('melanophages') in the upper dermis. The melanophages may also phagocytize other material such as lipofuscins and lipoproteins, thus producing a mixture which after denaturation may give unexpected staining results. Pathological deposition of melanin occurs in a benign lesion called a nevus or 'mole'. The malignant counterpart to the nevus is the malignant melanoma; it is in the diagnosis of this important tumor and its metastases that histological demonstration of melanin finds its most important practical application (Fig. 14.5). Melanin is also found in the hair follicles of dark-haired people.
2. *Eye*, where it is found normally in the choroid, ciliary body and iris. Similar brown-black pigment is found in the retinal epithelium but its identity with melanin is uncertain. Melanomas can occur in the eye but these tumors are rare.
3. *Brain*, where it is found particularly in the substantia nigra in such quantities that this structure is macroscopically visible as a black streak on both sides of the mesencephalon. In patients with long-standing Parkinson's disease this area is markedly reduced. Black melanin is also found in patches in the arachnoid covering some human brains, and has been described as having a 'sooty' appearance.

Demonstration of melanin

A number of methods can be used for the identification of melanin and melanin-producing cells. The most reliable of these are:

1. Reducing methods such as the Masson-Fontana silver technique and Schmorl's ferric-ferricyanide reduction test.
2. Enzyme methods e.g. DOPA reaction.
3. Solubility and bleaching methods.
4. Fluorescent methods.
5. Immunohistochemistry.

Melanin and its precursors are capable of reducing both silver and acid ferricyanide solutions. It also shows the marked physical property of being completely insoluble in most organic solvents which is almost certainly due to the fact that formed melanin is tightly bound to protein within the melanosome. The other physical characteristic shown by melanin is its ability to be bleached by strong oxidizing agents. This property is particularly useful when trying to identify nuclear detail in heavily pigmented melanocytic tumors. Further reference to these procedures will follow the conventional demonstration techniques for melanin. These two physical characteristics relate to formed melanin and not to melanin precursors.

The enzyme tyrosinase can be demonstrated by the DOPA reaction and is demonstrable in any cell capable of synthesizing melanin. Cells which have produced an abundance of melanin, and in which the melanosomes are filled with pigment, are said to no longer show tyrosinase activity, but some workers have found that tyrosinase is active in most cells, even though melanin may be present in large quantities.

The fluorescent method depends upon the ability of certain biogenic amines, including DOPA and dopamine, to show fluorescence after exposure to formaldehyde (formalin-induced fluorescence). This method therefore demonstrates melanin precursors rather than formed melanin. Recent advances in antibody production have produced a wide range of antibodies which recognize antigens in the melanin synthesis pathway, e.g. tyrosinase or tyrosinase related protein 1 and 2 (TRP 1 and 2), or those recognizing melanocyte activation antigens, e.g. gp100 (HMB 45) and Mart-1 (Melan A). The use of enzyme histochemical procedures is now rarely required.

Reducing methods for melanin

Melanin is a powerful reducing agent and this property is used to demonstrate melanin in the following ways:

1. The reduction of ammoniacal silver solutions to form metallic silver without the use of an extraneous reducer is known as the argentaffin reaction. Masson's (1914) method (using Fontana's silver solution) and its various modifications, which also rely on melanin's argentaffin properties, are now widely used for routine purposes. Melanins are blackened by acid silver nitrate solutions. Melanin is also argyrophilic, meaning that melanin is colored black by silver impregnation methods which use an extraneous reducer (Fig. 14.6). This is not a property considered to be of diagnostic value.
2. Melanin will reduce ferricyanide to ferrocyanide with the production of Prussian blue in the presence of ferric salts (the Schmorl reaction). This type of reaction (Fig. 14.7) is also seen with certain other pigments e.g. some lipofuscins, bile and neuroendocrine cell granules.
3. Other reducing methods for demonstrating melanin are Lillie's ferrous ion uptake (described in Lillie & Fullmer, 1976) and Lillie's Nile blue A (1956).

Masson-Fontana method for melanin (Fontana, 1912; Masson, 1914)

Fixation

Formalin is best; chromate and mercuric chloride should be avoided.

Sections

Works on all types of section, although some adjustment may be necessary for resin sections.

Preparation of silver solution (after Fontana)

Place 20 ml of a 10% aqueous silver nitrate solution in a glass flask. Using a fine-pointed dropper pipette, add concentrated ammonia drop by drop, constantly agitating the flask until the formed precipitate *almost* dissolves. This titration is critical if the method is to work consistently well. The end point of the titration is seen when a faint opalescence is present, and is best viewed using reflected light against a black background. If too much ammonia is inadvertently added, then the addition of a few drops of 10% silver nitrate will restore the opalescence. To this correctly titrated solution add 20 ml triple distilled water and then filter into a dark bottle. Store the solution in the refrigerator and use within 4 weeks, but note that ammoniacal silver solutions are potentially explosive if stored incorrectly.

Method

1. Take test and control sections to distilled water.
2. Treat with the ammoniacal silver solution in a Coplin jar which has been covered with aluminum foil, for 30–40 minutes at 56°C or overnight at room temperature.
3. Wash well in several changes of distilled water.
4. Treat sections with 5% aqueous sodium thiosulfate (hypo) for 1 minute.
5. Wash well in running tap water for 2–3 minutes.
6. Lightly counterstain in 0.5% aqueous neutral red or 0.1% aqueous nuclear fast red for 5 minutes.
7. Rinse in distilled water.
8. Dehydrate, clear and mount in a synthetic resin.

Results

Melanin, argentaffin, chromaffin and some lipofuscins	black
Nuclei	red

Notes

a. Only use thoroughly clean glassware, as the silver solution will react with any residual contaminant left on glassware.

b. Prolonged exposure to 56°C may give rise to a fine deposit over the section.

c. Friable material may need to be coated with celloidin as the ammonia in the silver solution could lead to sections lifting off the slide.

Microwave ammoniacal silver method for argentaffin and melanin (Churukian, 2005) (Fig. 14.6)

Fixation

10% buffered neutral formalin.

Sections

Paraffin wax embedded.

Preparation of solutions

Ammoniacal silver

To 10 ml of 2% silver nitrate add 5 ml of 0.8% lithium hydroxide monohydrate. Then add 28% ammonium hydroxide, drop by drop with constant shaking, until the precipitate almost dissolves. Make up the solution to 200 ml with distilled water and store in a refrigerator at 3–6°C. The solution is stable for at least 1 month.

0.2% aqueous gold chloride
2% aqueous sodium thiosulfate

Method

1. Take slides to distilled water.
2. Place slides in 40 ml of refrigerated cold ammoniacal silver solution in a plastic Coplin jar and microwave at a power setting of 360 W for 35 seconds. Gently agitate the Coplin jar for about 15 seconds. Microwave again at the same power for 35 seconds. Gently agitate the Coplin jar for about 15 seconds. Allow the slides to remain in the hot solution (approximately 80°C) for 2–3 minutes or until the sections appear a light brown.
3. Rinse in four changes of distilled water.
4. Place slides in 0.2% aqueous gold chloride for 1 minute.
5. Rinse in two changes of distilled water.
6. Place slides in 2% aqueous sodium thiosulfate for 1 minute.
7. Rinse in four changes of distilled water.
8. Counterstain with 0.1% aqueous nuclear fast red for 3 minutes.
9. Rinse in three changes of distilled water.
10. Dehydrate, clear, and mount in synthetic resin.

Results

Melanin, argentaffin, chromaffin, lipofuscin and other silver reducing substances	black
Nuclei	red

Notes

The results obtained with this method are similar to those obtained with the Masson-Fontana technique.

a. When preparing the ammoniacal silver solution, care must be taken not to add too much ammonium hydroxide. Add just enough to almost dissolve the precipitate.

b. The microwave oven used in this method had a maximum output of 600 watts with multiple power settings. Microwave ovens of higher or lower wattage may be used for this method by varying the microwave exposure times.

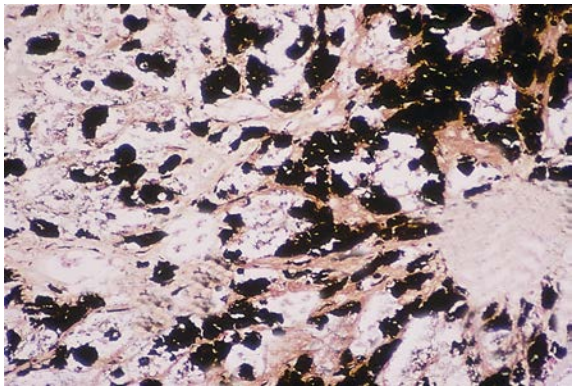


Fig. 14.6 A section of liver from a patient with malignant metastatic melanoma stained with Churukian's method for melanin. Melanin is stained black.

Schmorl's reaction (taken from Lillie, 1954) (Fig. 14.7)

Fixation

10% buffered neutral formalin.

Ferric-ferricyanide solution

Freshly prepared 0.4% aqueous potassium ferricyanide	4 ml
Freshly prepared 1% aqueous ferric chloride (or 1% ferric sulfate)	30 ml

N.B. Use this solution soon after mixing.

Method

1. Take test and control sections to distilled water.
2. Treat sections with the ferric-ferricyanide solution for 5–10 minutes.
3. Wash well in running tap water for several minutes to ensure that all residual ferricyanide is completely removed from the section.
4. Lightly counterstain with 0.5% aqueous neutral red or 0.1% aqueous nuclear fast red for 5 minutes.
5. Dehydrate, clear and mount in synthetic resin.

Results

Melanin, argentaffin cells, chromaffin, some lipofuscins, thyroid colloid and bile	dark blue
Nuclei	red

Notes

a. This modification is preferred to the more traditional method because it is easier to control and gives less background staining.

b. The time for the reaction to take place depends on the substance to be demonstrated, with melanin generally reacting faster than lipofuscin. This fact should not be taken as a definitive diagnostic pointer but only as a general guideline.

c. When choosing a control section, it is important to remember that melanin reduces the ferric-ferricyanide faster than other reducing substances. Therefore, control sections should always match the test sections so that a lipofuscin control should not be used if the test pigment is thought to be melanin.

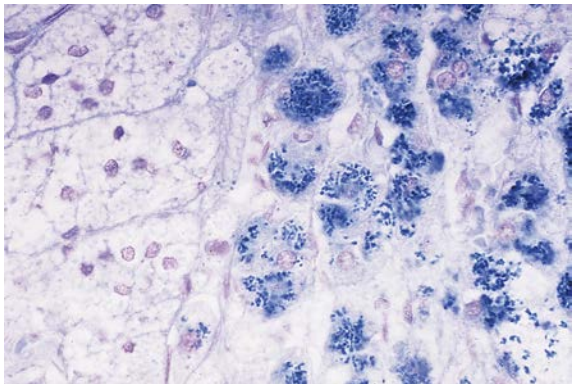


Fig. 14.7 A section of adrenal stained with Schmorl's method for reducing substances. Chromaffin is stained blue.

Enzyme methods for melanin

Cells which are capable of producing melanin can be demonstrated by the DOPA oxidase method. The enzyme tyrosinase which is localized within these cells will oxidize DOPA to form an insoluble brown-black pigment. The best results are obtained when using post-fixed cryostat sections, although a useful, but less reliable method uses freshly fixed blocks of tissue.

These methods are those of Bloch (1917) and Laidlaw and Blackberg (1932) for tissue sections, and Bloch (1917) and Rodriguez and McGavran (1969) for tissue blocks. Though previously included in the fifth edition of this book, these methods are currently not in use.

Solubility and bleaching methods for melanin

Melanins are insoluble in most organic solvents or in anything which will significantly destroy the tissue which contains them. The insolubility shown by melanin is due to the tight bond it has with its protein component. Use of strong oxidizing agents, such as permanganate, chlorate, chromic acid, peroxide and peracetic acid, will bleach melanin. The blacker the melanin, the longer the bleach takes to decolor, or bleach the pigment. Lipofuscin tends to take longer to be bleached from paraffin sections than melanin. The method of choice is peracetic acid, but treatment with 0.25% potassium permanganate followed by 2% oxalic acid also works well. The difficulty with these procedures is that they can have detrimental effects on the quality of the tissue section following the oxidation steps. In addition, the oxidation can also damage antigenic binding sites for subsequent immunohistochemical investigations. One of the methods of choice is dilute hydrogen peroxide bleaching. Certainly in the investigation of ophthalmic pathology this method has proved popular (Kivela, 1995). In cutaneous pathology a method introduced by Orchard (1999, 2007) involving the use of 10% diluted hydrogen peroxide made in phosphate buffered saline (PBS pH 7.6) and incubated in a water bath or oven at 60°C works effectively, with complete bleaching being achieved within 1 hour in the majority of cases. The key benefit of this is that it does not destroy or damage antigenic epitopes and therefore allows an extensive panel of immunohistochemical antibody investigations. Immunohistochemistry is often required in such cases, since the distinction between melanocytes and melanophages (melanin-containing macrophages) is often critical. A good example of this is the need to distinguish between cases of malignant melanoma and dysplastic nevi with extensive dermal melanosis (Figs. 14.8a, b and c). This distinction is based on the use of a selective melanocyte antibody, such as HMB 45 (Fig. 14.8d) or Melan A and an antibody which recognizes macrophages, e.g. CD68 (KP1) (Fig. 14.8e). This procedure becomes mandatory in cases where melanin deposits are so dense that the nuclear detail of the involved cells is totally obscured. The standard chromogen for immunohistochemical procedures is 3, 3-diaminobenzidine tetrahydrochloride (DAB), which produces a brown reaction product. The use of red chromogens, such as alkaline phosphatase, may enable clear visualization in some cases, although not often adequate to clearly see the immunohistochemical localization of the antigen to be demonstrated in cases of extensive dermal melanosis. Similarly, stains such as azure B can be used as proposed by Kamino and Tam (1991); it will stain the melanin pigment a green color in an immunohistochemical procedure.

Bleaching melanin pigment using hydrogen peroxide (H_2O_2) (Orchard, 2007)

Fixation

10% neutral buffered formalin.

Solution

40% H_2O_2	5 ml
Phosphate buffered saline (pH 7.6)	45 ml

(Dissolve PBS tablets according to instructions in 200 ml of distilled water per tablet.)

Make the solution fresh and place in a 50 ml Coplin jar.

Method

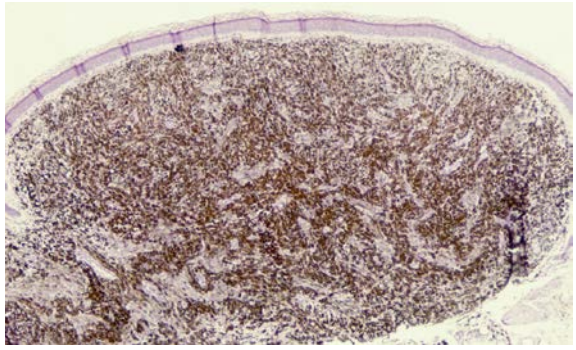
1. Take test and control sections to distilled water.
2. Prepare the incubating solution fresh and place in a water bath or oven at 60°C for 10 minutes.

3. Place slides into the incubating solution and ensure the Coplin jar lid is securely placed over the jar.
4. Incubate in the jar for 1 hour.
5. Remove slides and wash in running tap water for 3 minutes.
6. Continue with immunohistochemical procedures (heat-mediated or enzyme digestion antigen retrieval techniques).

Notes

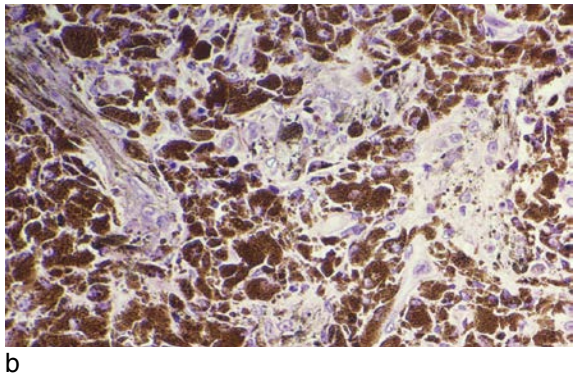
a. The melanin bleaching effect can be seen with the naked eye in 1 hour with most cases. In circumstances of excessive melanin deposition slightly longer incubation times may be required. The end point can be determined by removing slides, washing in tap water, and checking under the staining microscope to ensure completeness of the bleaching effect.

b. Heat-mediated antigen retrieval procedures can be performed according to normal immunohistochemical procedures; enzyme digestion techniques may require reductions in the time for digestion (normally half the usual exposure time).



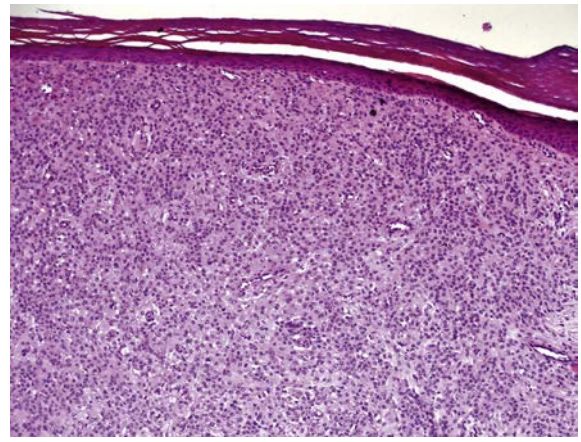
a

Fig. 14.8a A section of a dysplastic nevus with extensive dermal melanosis stained with H&E.



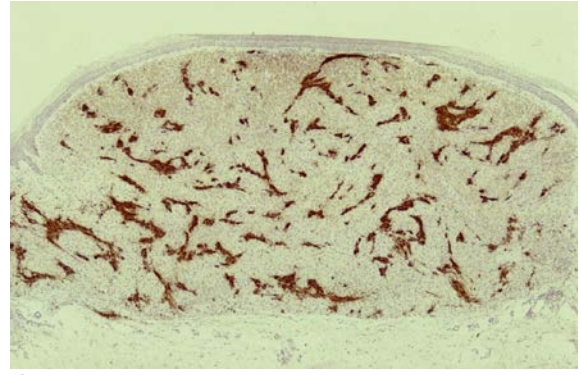
b

Fig. 14.8b The same case at higher magnification, showing extensive melanin deposition.



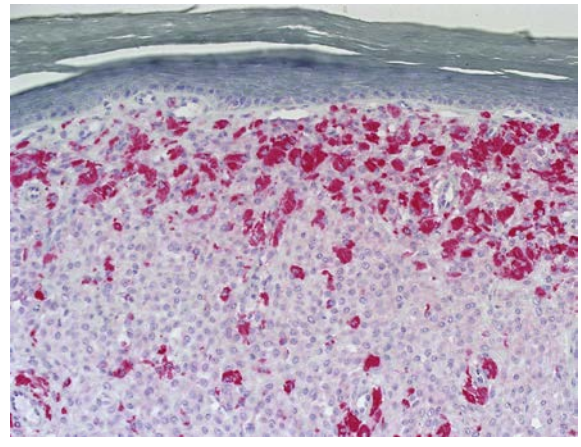
c

Fig. 14.8c The same case at the higher magnification bleached prior to H&E staining.



d

Fig. 14.8d The same case at lower magnification labelled with HMB 45 following melanin bleaching, demonstrating cords of residual melanocytes remaining following destruction of the lesion by the host immune response. The melanin-containing cells are melanophages.



e

Fig. 14.8e CD68 labelling following H_2O_2 bleaching in the same case as 14.8a–d. showing positive labelling (in red) of melanophages.

Formalin-induced fluorescence (FIF)

Certain aromatic amines such as 5-hydroxytryptamine, dopamine, epinephrine (adrenaline), norepinephrine (noradrenaline) and histamine, when exposed to formaldehyde, show a yellow primary fluorescence. This is particularly useful when demonstrating amelanotic melanoma because these tumors can be difficult to diagnose using conventional methods due to their lack of pigment. Any melanin precursors present will form a product of isocarboline derivatives which are dehydrogenated and will show yellow fluorescence. The best results are seen when using tissue which has been freeze-dried (see Chapter 7) and then fixed using paraformaldehyde vapor. Formalin-fixed frozen sections will give acceptable results, paraffin wax processed tissue can also be used but shows weak fluorescence which is difficult to visualize.

Formaldehyde-induced fluorescence method for melanin precursor cells (Eranko, 1955)

Fixation

10% buffered neutral formalin.

Sections

Cryostat or 5 µm paraffin wax sections.

Method

1. Deparaffinize sections in xylene. Fix frozen sections in 10% buffered formalin for 5 minutes, dehydrate and place in xylene.
2. Rinse in fresh xylene.
3. Mount in a medium which is fluorescence free.
4. Examine using a fluorescence microscope with BG38, UG1 and a barrier filter.

Results

Melanin precursor cells show weak yellow fluorescence.

Notes

a. A brighter image will be seen if epi-illumination is used.
b. Some commercially produced mountants are unsuitable as they fluoresce and will confuse the result.
c. One of the claims originally made about FIF was that archived paraffin-processed material could be examined to see if there was evidence of melanin precursor cells, but because of the indifferent results it is not widely used.

Other methods for melanin

Ferrous ion uptake reaction for melanin (Lillie & Fullmer, 1976) (Fig. 14.9)

Fixation

Formalin is best; avoid all chromate fixatives.

Sections

Paraffin wax embedded.

Solutions

2.5% ferrous sulfate
1% potassium ferricyanide in 1% acetic acid

Method

1. Take test and control sections to distilled water.
2. Place in 2.5% ferrous sulfate for 1 hour.
3. Wash with six changes of distilled water.
4. Place in 1% potassium ferricyanide in 1% acetic acid for 30 minutes.
5. Wash with four changes of distilled water.
6. Counterstain with 0.5% aqueous neutral red or 0.1% nuclear fast red.
7. Rinse in two changes of distilled water.
8. Dehydrate, clear and mount in synthetic resin.

Results

Melanins and neuromelanin	dark green
Nuclei	red

Notes

a. According to Lillie this method is specific for melanin.
b. Ferric iron and lipofuscins do not stain with this method.

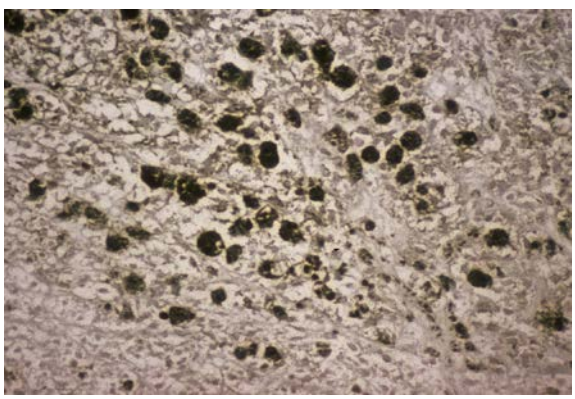


Fig. 14.9 A section of liver from a patient with malignant melanoma stained with Lillie's ferrous ion uptake method for melanin. Melanin is stained black.

Nile blue method for melanin and lipofuscin
(Lillie, 1956) (Figs. 14.10 and 14.11)

Fixation

10% buffered neutral formalin.

Sections

Paraffin wax embedded.

Nile blue solution

Nile blue (CI 51180)	0.05 g
Distilled water	99 ml
Sulfuric acid	1.0 ml

Dissolve Nile blue in distilled water then add sulfuric acid.

Method

1. Take test and control sections to distilled water.
2. Place in Nile blue solution for 20 minutes.
3. Wash with four changes of distilled water.
4. Mount in an aqueous mountant (e.g. glycerin jelly).

Results

Melanin	dark blue
Lipofuscin	dark blue
Nuclei	blue or unstained

Notes

a. Some samples of Nile blue may not yield satisfactory results with this method.
b. Using frozen sections, this method will stain neutral lipids (triglycerides, cholesterol esters and steroids) red to pink. Acidic lipids (fatty acids and phospholipids) stain blue.

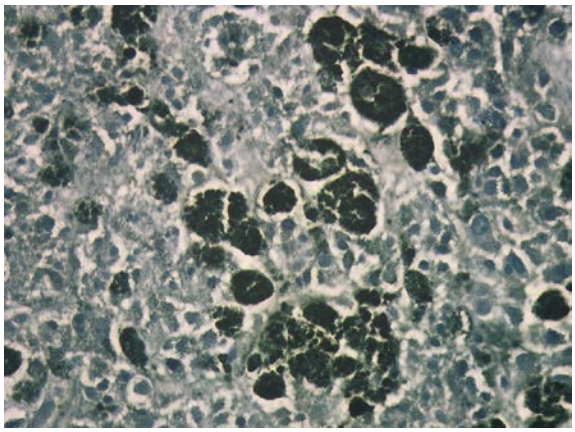


Fig. 14.10 A section of liver from a patient with malignant melanoma stained with Lillie's Nile blue method for melanin. Melanin is stained dark blue.

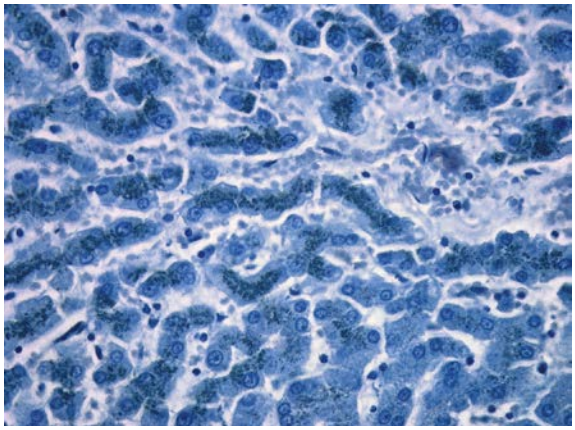
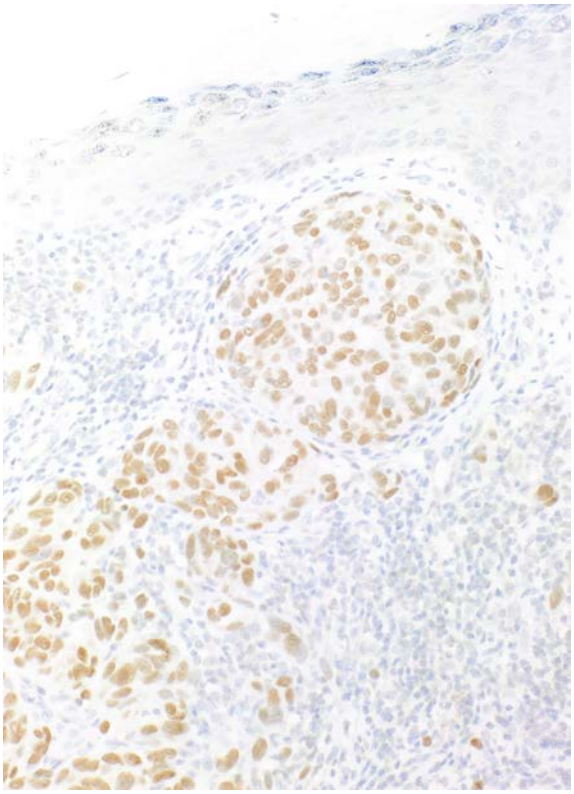


Fig. 14.11 A section of liver stained with Lillie's Nile blue method for lipofuscin. Lipofuscin within the hepatocytes is stained dark blue.

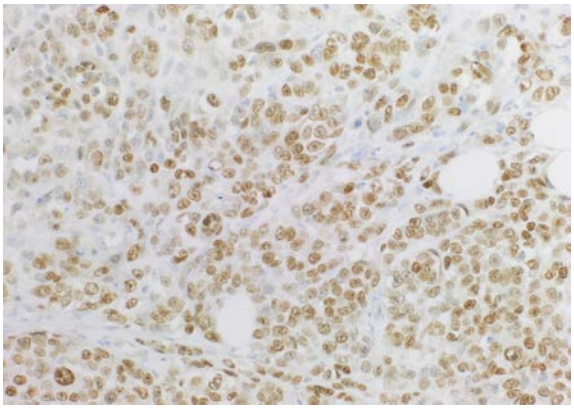
Immunohistochemistry

Brief mention should be given to the use of melanocyte selective antibodies to highlight melanocytic lesions. The large majority of these antibodies recognize an antigen associated with melanocyte activation, gp100 (HMB 45) or Mart-1 (Melan A). Other selective antibodies such as tyrosinase are directly linked to antigens associated with melanin synthesis. There is a new antibody called Sox-10 which has recently become available. Sox-10 is from the Sry-related HMG-Box gene 10 (Sox-10). It is a nuclear transcription factor involved in the neural crest development of cells of melanocytic lineage. It therefore has a nuclear labelling profile. It is a highly sensitive marker for malignant melanoma, but it does not distinguish between malignant and benign melanocytes. In addition, it will label Schwann cells, myoepithelial cells (salivary, bronchial and mammary gland), eccrine glands and mast cells. It is positive in the majority of primary and metastatic melanomas with reports of positive labelling in over 97% of melanomas tested (Fig. 14.12a, b). It is also highly effective on unusual variants of malignant melanoma such as desmoplastic malignant melanoma with positive labelling rates of between 74-100% of cases tested. These antibodies are highly valuable in cases of delineating atypical



a

Fig. 14.12a Sox-10 labelling in a case of primary cutaneous superficial spreading malignant melanoma.



b

Fig. 14.12b High power view of Fig. 14.12a showing clear nuclear labelling of malignant melanocytes.

melanocytic lesions from a host of other tumor types, particularly if the lesions are amelanotic. The histological and morphological appearances of malignant melanomas can be highly variable, with epithelioid, spindle or even small round cell appearances of the tumor cells. There are currently no antibodies which can reliably distinguish between malignant and benign melanocytic lesions for use in routine histopathology. The 'gold standard' antibody is now Sox-10, as it will label the majority of melanocytic lesions. However, this antibody recognizes an antigen expressed in cells derived from the neural crest and, as such, is not specific for melanocytes alone. In difficult cases a panel of antibodies is often applicable (Orchard, 2000; Ordonez, 2014; Sommer, 2011).

Lipofuscins

These yellow to red-brown pigments occur widely throughout the body and are thought to be produced by an oxidation process of lipids and lipoproteins. The oxidation process occurs slowly and progressively, and therefore the pigments exhibit variable staining reactions, different colors, and variation in shape and size which appears to be dependent upon their situation. This type of pigment is found in the following sites:

- Hepatocytes, sometimes as a mixture with other types of pigment.
- Cardiac muscle cells, particularly around the nucleus. Large amounts of pigment are found in the small brown hearts of elderly debilitated people, a condition known as 'brown atrophy of the heart'.
- Inner reticular layer of the normal adrenal cortex, where the pigment imparts a brown color and is particularly prominent in patients dying after a long and stressful illness.
- Testis, particularly in the interstitial cells of Leydig. It is responsible for giving testicular tissue its brown color.
- Ovary, in the walls of involuting corpora lutea and in some macrophages around the corpora lutea. Hemosiderin tends to be seen more commonly in this situation.
- Cytoplasmic inclusions in the neurons of the brain, spinal cord and ganglia.
- The edge of a cerebral hemorrhage or infarct.

- Some lipid storage disorders such as Batten's disease.
- Other tissues such as bone marrow, involuntary muscle, cervix and kidney.

Demonstration of lipofuscins

It is important to bear in mind that because lipofuscin is formed by a slow progressive oxidation process of lipids and lipoproteins, histochemical reactions will vary according to the degree of oxidation present in the pigment when the demonstration techniques are applied. Therefore, it is advisable to carry out a variety of techniques in order to be sure whether the pigment is lipofuscin. The lipofuscins react with a variety of histochemical and tinctorial staining methods, the most common and useful being:

- Periodic acid-Schiff method (page 183).
- Schmorl's ferric-ferricyanide reduction test (page 209).
- Long Ziehl-Neelsen method (see below).
- Sudan black B method (page 498).
- Gomori's aldehyde fuchsin technique (see below and Fig. 14.13).
- Masson-Fontana silver method (page 207).
- Basophilia, using methyl green.
- Churukian's silver method (page 208).
- Lillie's Nile blue sulfate method (page 213 and Fig. 14.11).

Long Ziehl-Neelsen method (Pearse, 1953)

Fixation

Any routine fixative.

Sections

Works well on all types of tissue section.

Method

1. Take slides to distilled water.
2. Stain in a Coplin jar with filtered carbol fuchsin using a 60°C water bath for 3 hours, or overnight at room temperature.
3. Wash well in running water.
4. Differentiate in 1% acid alcohol until the background staining is removed.
5. Wash well in running tap water.
6. Counterstain nuclei with 0.25% aqueous methylene blue in 1% aqueous acetic acid for 1 minute.
7. Dehydrate, clear and mount in synthetic resin.

Results

Lipofuscin	magenta
Ceroid	magenta
Nuclei	blue
Background	pale magenta to pale blue

Notes

a. Experience has shown that a more reliable result is obtained if the staining is carried out using a thermostatically controlled water bath.

b. A useful variant of this method is cited by Lillie (1954), in which the staining solution is modified so that Victoria blue is substituted for basic fuchsin. It is sometimes difficult to distinguish between the red of the fuchsin and the red-brown of the pigment, whereas the blue color of lipofuscin with the Victoria blue may be more convincing.

Aldehyde fuchsin technique (Gomori, 1950) (Fig. 14.13)

Fixative

10% buffered neutral formalin.

Sections

Paraffin wax embedded.

Solutions

Acidified potassium permanganate solution

(0.25% aqueous potassium permanganate in 0.1% sulfuric acid)
2% aqueous oxalic acid

Aldehyde fuchsin

Dissolve 1 g pararosanilin (CI 42500) in 100 ml aqueous 70% ethanol. Add 1 ml concentrated hydrochloric acid and 2 ml paraldehyde or acetyl-aldehyde, shaking the mixture thoroughly. Stand for 3–5 days at room temperature or preferably longer, near natural light, to allow the solution to blue. Store the solution at 4°C. The solution will remain viable for approximately 2 months. Any increase in the background staining will indicate deterioration of the staining solution.

Method

1. Take sections to distilled water.
2. Treat with acidified potassium permanganate solution for 5 minutes.
3. Wash well in distilled water and treat for 2 minutes with oxalic acid solution to bleach section.
4. Wash well in distilled water.

5. Rinse in 70% ethyl alcohol.
6. Stain section in aldehyde fuchsin for 5 minutes. Longer staining times will be needed as the solution ages.
7. Rinse in 70% ethyl alcohol followed by a rinse in three changes of distilled water.
8. Dehydrate, clear and mount in synthetic resin.

Results

Lipofuscin	purple
Elastic fibers	purple

Notes

a. Paraldehyde should be freshly opened (Moment, 1969). It keeps well when stored in the freezing compartment of a refrigerator. If paraldehyde is not available, acetaldehyde may be used and need not be refrigerated.

b. Other tissue constituents, such as beta cells of the pancreas and pituitary, elastin, sulfated mucins, gastric chief cells and neurosecretory granules, will stain with this method.

c. The basic fuchsins, rosanilin and new fuchsin are closely related to pararosanilin. Only pararosanilin will give satisfactory staining results in this procedure.

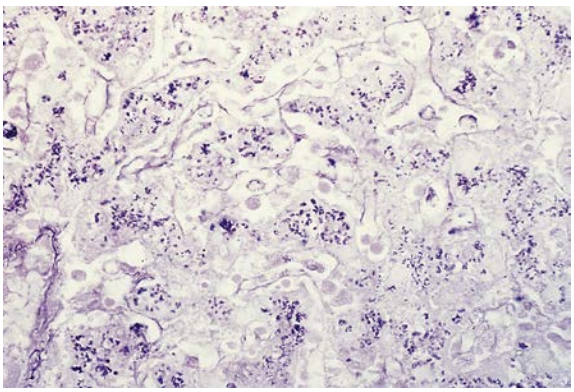


Fig. 14.13 A section of liver stained with Gomori's aldehyde fuchsin. Lipofuscin is stained purple.

Chromaffin

This pigment is normally found in the cells of the adrenal medulla as dark brown, granular material. It may occur in tumors of the adrenal medulla (pheochromocytomas).

Fixation in formalin is not recommended, and fixatives containing alcohol, mercury bichloride or acetic acid should be avoided. Orth's or other dichromate-containing fixatives are recommended. Chromaffin may be demonstrated by Schmorl's reaction (Fig. 14.7), Lillie's Nile blue A, the Masson-Fontana, Churukian's microwave ammoniacal silver method and the periodic acid-Schiff (PAS) technique.

Pseudomelanosis pigment (melanosis coli)

This pigment is sometimes seen in macrophages in the lamina propria of the large intestine and appendix. Various theories have been put forward as to its nature. The current view is that it is an endogenous lipopigment whose reactions are those of a typical ceroid-type lipofuscin. It appears to be strongly associated with anthraquinone purgatives ('cascara sagrada'). Its distinction from melanin may occasionally be important. Pseudomelanosis will, in general, stain with those methods which are used to demonstrate lipofuscin e.g. Masson-Fontana and Schmorl.

Dubin-Johnson pigment

This pigment is found in the liver of patients with Dubin-Johnson syndrome and is due to defective canalicular transport of bilirubin. It is characterized by the presence of a brownish-black, granular, intracellular pigment situated in the centrilobular hepatocytes. The true nature of the pigment has yet to be established, but histochemically it is similar to lipofuscin, though there are ultrastructural differences.

Ceroid-type lipofuscins

Lillie et al. (1941, 1942) were the first to describe ceroid in the cirrhotic livers of animals maintained on inadequate diets. Lillie thought that ceroid was different from lipofuscin because it failed to stain with the ferric-ferricyanide reaction. Pearse (1985) states that ceroid is in fact a lipofuscin at an early stage of oxidation. Further oxidation would produce lipofuscin proper.

Hamazaki-Weisenberg bodies

These small, yellow-brown, spindle-shaped structures are found mainly in the sinuses of lymph nodes, either lying free or as cytoplasmic inclusions, and their significance is unknown. First

described by Hamazaki (1938), they have been described as being present in lymph nodes from patients with sarcoidosis (Weisenberg 1966). Further studies have shown them to be present in a number of conditions (Boyd & Valentine 1970). Hall and Eusebi (1978) have reported their presence in association with melanosis coli. Histochemically they are similar to lipofuscin, and at ultrastructural level have an appearance which suggests that they are probably giant lysosomal residual bodies (Doyle et al. 1973).

Endogenous minerals

Iron is discussed under the heading 'Hematogenous pigments'.

Calcium

Insoluble inorganic calcium salts are a normal constituent of bones and teeth, and their demonstration is well covered in Chapter 17. From a histochemical viewpoint, the free ionic form of calcium, found in blood, cannot be demonstrated. Abnormal depositions of calcium can be found in necrotic areas of tissue associated with tuberculosis, infarction (Gandy-Gamna bodies), atheroma in blood vessels and malakoplakia of the bladder (Michaelis-Gutman bodies). The most common forms of calcium salts occurring in these conditions are phosphates and carbonates. Calcium salts are usually monorefringent but calcium oxalate is birefringent (Fig. 14.14). Calcium usually stains purple-blue with H&E.

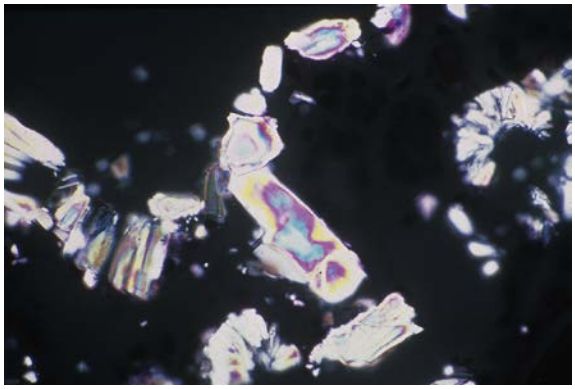


Fig. 14.14 A section of kidney with calcium oxalate crystals as seen with polarization microscopy.

The use of various dyes which act by forming chelate complexes with calcium has been practiced by many workers. These dyes include alizarin red S, purpurin, naphthochrome green B and nuclear fast red. In general, these dyes demonstrate medium to large amounts of calcium better than particulate deposits which stain weakly, the exception being alizarin red S, which tends to give more reliable results with small deposits. None of these dyes is specific to calcium salts, although alizarin red S when used at pH 4.2 is so considered. The classic method of von Kossa (1901), which uses silver nitrate, is generally preferred for routine demonstration purposes on paraffin sections (Fig. 14.15).

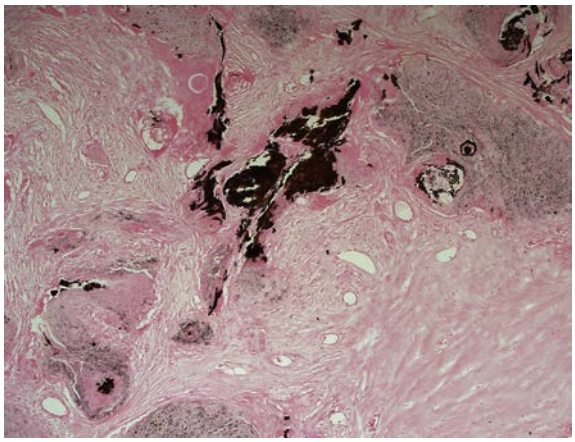


Fig. 14.15 A section of skin in a case of a dermal cyst stained with von Kossa's silver nitrate method for calcium. An ultraviolet lamp was used to perform the method. Calcium is stained black.

This method demonstrates only phosphate and carbonate radicals, giving good results with both large and small deposits of calcium. The method is not specific, as melanin will also reduce silver to give a black deposit. As a general rule, fixation of tissue containing calcium deposits is best when using non-acidic fixatives such as buffered neutral formalin, formal alcohol or alcohol.

Alizarin red S method for calcium (Dahl, 1952; McGee-Russell, 1958; Luna, 1968)

Fixation

Buffered neutral formalin, formal alcohol and alcohol.

Sections

Paraffin wax or frozen.

Solutions

1% aqueous alizarin red S (CI 58005) adjusted to pH 4.2 or pH 6.3–6.5 with 10% ammonium hydroxide.
0.05% fast green FCF (CI 42053) in 0.2% acetic acid.

Method

1. Take sections to 95% alcohol.
2. Stand slides on end and thoroughly air dry.
3. Place sections in a Coplin jar filled with the alizarin red S solution for 5 minutes (see Notes below).
4. Rinse quickly in distilled water.
5. Counterstain with fast green for 1 minute.
6. Rinse in three changes of distilled water.
7. Dehydrate, clear and mount in synthetic resin.

Results

Calcium deposits	orange-red
Background	green

Notes

a. The staining time is dependent on the amount of calcium present.
b. Calcium deposits are birefringent after staining with alizarin red S.
c. McGee-Russell recommends using the alizarin red S at pH 4.2. Dahl indicated that it be used at pH 6.36 to pH 6.4. Churukian is in agreement with Dahl as to the higher pH and has observed that a pH as high as 7.0 produces good results.
d. This method is particularly useful in the identification and detection of small amounts of calcium as seen in heterotopic calcification in the kidney (hypercalcinosis). This type of tissue also makes excellent control material (Fig. 14.16).

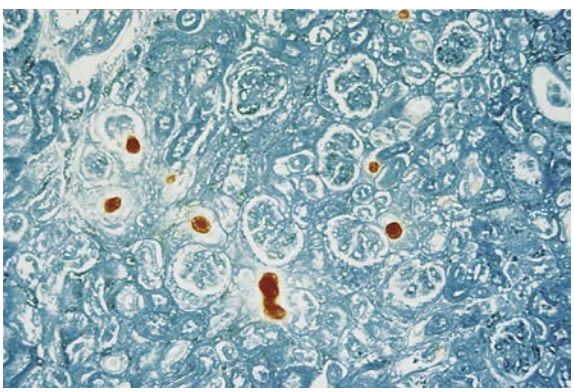


Fig. 14.16 A section of kidney stained with Dahl's alizarin red S method at pH 6.4. Calcium is stained orange-red.

Copper

Many enzymes in the body would fail to function without the presence of copper, although copper deficiency is extremely rare. Copper accumulation is associated with Wilson's disease, the most important disorder of copper metabolism. This disease is a rare, inherited, autosomal recessive condition which gives rise to copper deposition in the liver, basal ganglia of the brain and eyes. In the eye, the Kayser-Fleischer ring, a brown ring of deposited copper, may be seen in the cornea (Descemet's membrane), and is diagnostic of this condition. Copper deposition in the liver is also associated with primary biliary cirrhosis and certain other hepatic disorders.

Copper, like many metallic cations, is capable of forming a blue dye lake using Mallory's unripened hematoxylin. Uzman (1956) modified the Okamoto-Utamura rubeanate method (1938) and obtained excellent results on formalin-fixed tissue. The rhodanine method (Lindquist, 1969) has also been used to demonstrate copper and copper-associated protein (CAP). Although the quality of the reagent, dimethylaminobenzylidine rhodanine (DMABR), varies considerably, it is considered to be the method of choice. CAP is also well demonstrated by the Shikata orcein method (Shikata et al., 1974).

Rubeanic acid method for copper (Okamoto & Utamura, 1938; Uzman, 1956)

Fixative

10% buffered neutral formalin.

Rubeanic acid solution

0.1% rubeanic acid (dithio-oxamide) in absolute ethyl alcohol	5 ml
10% aqueous sodium acetate	100 ml

Prepare fresh before use.

Method

1. Take the test section, together with a known positive control section, to distilled water.
2. Place sections in a Coplin jar filled with rubeanic-acetate solution for at least 16 hours at 37°C. Times may need to be extended and the method is best carried out in a water bath.
3. Wash in 70% ethyl alcohol.
4. Rinse briefly in distilled water.
5. Drain section and blot dry.

6. Lightly counterstain with 0.5% aqueous neutral red or 0.1% aqueous nuclear fast red for 1 minute.
7. Rinse in distilled water.
8. Dehydrate, clear, and mount in synthetic resin.

Results

Copper	greenish black
Nuclei	pale red

Notes

a. Correct choice of fixative is essential. Buffered neutral formalin is acceptable, but avoid the use of acid formalin and fixatives containing mercury and chromium salts.
b. To demonstrate any copper which is bound to CAP, deparaffinized sections are placed downwards over a beaker of concentrated hydrochloric acid for 15 minutes. The sections are washed well in absolute alcohol and then transferred to the rubeanic-acetate solution.
c. The method is best carried out using a thermostatically controlled water bath.

Modified rhodanine technique (Lindquist, 1969)

Fixative

10% buffered neutral formalin.

Sections

Paraffin wax.

Solutions

Rhodanine stock solution

5-*p*-Dimethylaminobenzylidene-rhodanine	0.05 g
Absolute ethanol	25 ml

Prepare fresh and filter prior to use.

Working solution

5 ml of the stock rhodanine solution added to 45 ml of 2% sodium acetate trihydrate.

Borax solution

Disodium tetraborate	0.5 g
Distilled water	100 ml

Method

1. Take test and control sections to water.
2. Incubate in the rhodanine working solution at 56°C for 3 hours or overnight in a 37°C oven.
3. Rinse in several changes of distilled water for 3 minutes each.
4. Stain in acidified Lillie-Mayer or other alum hematoxylin for 10 seconds.
5. Briefly rinse in distilled water and place immediately in borax solution for 15 seconds.
6. Rinse well in distilled water.
7. Mount with Apathy's mounting media.

Results

Copper and copper-associated protein	red to orange-red
Nuclei	blue
Bile	green

Notes

a. Certain synthetic mountants will cause fading of the copper and CAP in archived material. No fading occurs when sections are mounted with Apathy's media. This method will give the most consistent results when in the hands of an experienced practitioner.
b. Results help distinguish between bile and iron pigments (Irons et al., 1977).
c. Analytical grade reagents and triple distilled water are recommended when performing this technique.
d. Control material is best obtained from livers of patients suffering from Wilson's disease, primary biliary cirrhosis or other forms of chronic cholestasis. Fetal liver of the third trimester (Fig. 14.17) fixed in buffered neutral formalin for not more than 36 hours makes a good positive control.

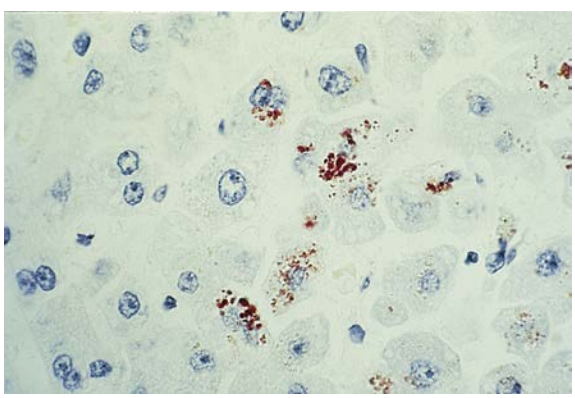
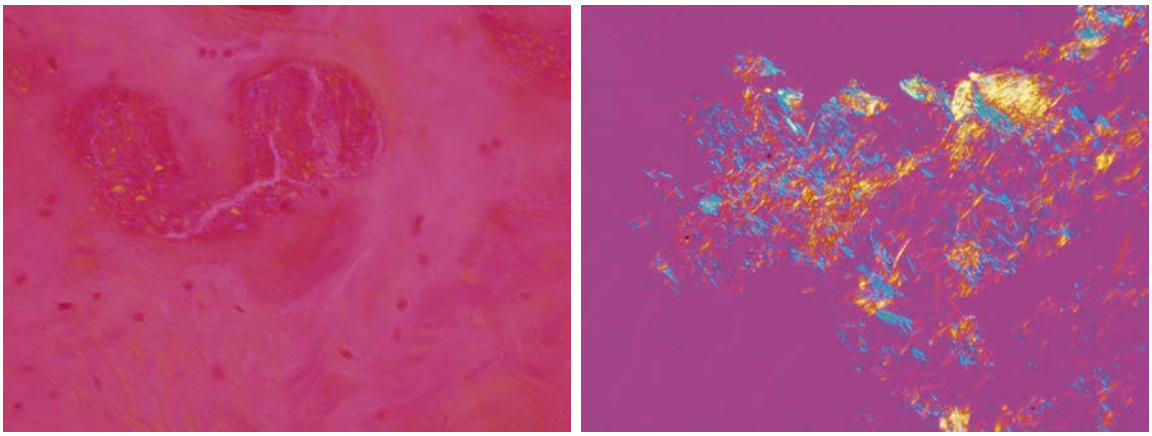


Fig. 14.17 A section of fetal liver of the third trimester stained with Lindquist's method for copper. Copper is stained red to orange-red.



a b

Fig. 14.18 Visualized through a quartz first-order red compensator, small, rather brick-like/oblong pyrophosphate crystals (pseudogout) exhibit a positive birefringence **(a)**, contrasting with the needle-shaped urate crystals **(b)** which show a negative birefringence.

Uric acid and urates

Uric acid is a breakdown product of the body's purine (nucleic acid) metabolism, although a small proportion is obtained from the diet. Most, but not all uric acid is excreted by the kidneys. The uric acid circulating in the blood is in the form of monosodium urate, which in patients with gout may be high, forming a supersaturated solution. These high levels may result in urate depositions, which are water soluble in tissues, causing:

- Subcutaneous nodular deposits of urate crystals known as 'tophi'
- Synovitis and arthritis
- Renal disease and calculi.

Another condition which occasionally can mimic gout is known as *pseudogout* or *chondrocalcinosis*, and is a pyrophosphate arthropathy. This results in calcium pyrophosphate crystals being deposited in joint cartilage. The cause of this deposition is unknown and is more common in the elderly, affecting mainly the large joints, such as the knee. It is important that gout and pseudogout are distinguishable.

To aid the diagnosis, a polarizing microscope fitted with a quartz first-order red compensator will prove useful, although sellotape applied in a single strip on a glass slide can occasionally suffice! Whilst pyrophosphate crystals exhibit a positive birefringence (Fig. 14.18a) as small cuboidal/oblong crystals often within the tissues, urate crystals show a negative birefringence with needle-shaped crystals (Fig. 14.18b). If sections have been prepared from routine formalin-fixed, paraffin-processed material, many crystals may have been leached out. Urates can be extracted by saturated aqueous lithium carbonate solution (Gomori, 1951), whilst pyrophosphate crystals are unaffected. If this extraction sequence is used in combination with Grocott's modification of Gomori's hexamine silver technique, both types of crystal can usually be identified.

Lithium carbonate extraction-hexamine silver technique (Gomori, 1936, 1951; Grocott, 1955)

Fixation

Urate crystals are water soluble, therefore fixation in alcohol will give a more specific reaction.

Sections

Paraffin wax, frozen or celloidin.

Solutions

Grocott's hexamine silver solution
Saturated aqueous lithium carbonate solution
2% aqueous sodium thiosulfate (hypo)
0.05% aqueous fast green (CI 42053) in 0.2% acetic acid

Method

1. Take two test sections and two control sections to 70% ethyl alcohol.
2. Place one section from each pair in saturated aqueous lithium carbonate solution for 30 minutes.
3. Rinse all sections in distilled water.
4. Place all sections in a Coplin jar filled with hexamine silver solution for 1 hour at 45°C.
5. Wash sections in distilled water.
6. Treat sections with hypo for 30 seconds.
7. Counterstain with fast green solution for 1 minute.
8. Wash in water, dehydrate, clear and mount in synthetic resin.

Results

Extracted sections	urates only are extracted
Unextracted sections	urates and possibly pyrophosphates are blackened
Background	green

Notes

a. The urates reduce the silver solution due to their argentaffin properties.

b. More accurate control of incubation temperature is achievable using a thermostatically controlled water bath.

Artifact pigments

This group of pigments comprises:

- Formalin
- Malaria
- Schistosome
- Mercury
- Chromic oxide
- Starch.

Formalin pigment

This pigment is seen as a brown or brown-black deposit in tissues which have been fixed in acidic formalin. The deposit is usually present in blood-rich tissues such as spleen, hemorrhagic lesions and large blood vessels filled with blood. The morphology of the pigment can vary but is commonly seen as a microcrystalline deposit which is anisotropic (birefringent). It is related to the acid hematins, but is spectroscopically distinct from hydrochloric acid and acetic acid hematins (Herschberger & Lillie, 1947).

One way of removing this pigment from tissue sections is by treating unstained tissue sections with saturated alcoholic picric acid. Alcoholic solutions of both sodium and potassium hydroxide will also remove the pigment but these may have deleterious effects on subsequent staining techniques. Treatment with 10% ammonium hydroxide in 70% alcohol for 5–15 minutes will remove this pigment and is less harmful to tissue sections than the other hydroxides. The use of buffered neutral formalin will help to minimize the problem of formalin pigment deposition. Fixation of large blood-rich organs, e.g. spleen for a long period will tend to increase the amount of formalin pigment formed. Under these conditions it is advisable to change the fixative on a regular basis.

Malarial pigment

This pigment is morphologically similar to formalin pigment and occasionally may be identical, even though it is produced in a slightly different manner. It is formed within, or in the region of, red blood cells which contain the malaria parasites (*Plasmodium malariae*, *ovale*, *vivax* and *falciparum*). In cases of cerebral malaria, due to infection with *Plasmodium falciparum*, malaria pigment can be seen in, or over, the red blood cells within the tiny blood capillaries of the brain. The presence of heavy erythrocyte parasitization areas provides the support for the diagnosis before confirmatory histochemistry. The pigment may, on occasion, be so heavily deposited that it obscures the visualization of the malaria parasite. Malaria pigment may also be present within phagocytic cells which have ingested infected red cells. Therefore, one should carefully examine the Kupffer cells of the liver, the sinus lining cells of lymph nodes and spleen, and phagocytic cells in the bone marrow. Malaria pigment, like formalin pigment, exhibits birefringence and can be removed from tissue sections with saturated alcoholic picric acid, but usually requires 12–24 hours treatment for complete removal.

Much less time is required to remove the pigment by using 10% ammonium hydroxide, as described below.

Extraction method for formalin and malaria pigment

Solutions

10% ammonium hydroxide in 70% ethyl alcohol.

Method

1. Take sections to 70% ethyl alcohol.
2. Place sections in a Coplin jar containing ammonium hydroxide in alcohol for 5–15 minutes.
3. Wash well in distilled water.
4. Apply staining method desired.

Notes

a. The time necessary for the removal of formalin pigment will vary, depending on the amount of pigment present.
b. Malaria pigment usually requires treatment for at least 15 minutes or longer.

Schistosome pigment

This pigment is occasionally seen in tissue sections where infestation with *Schistosoma* is present. The pigment, which tends to be chunky, shows similar properties to those of both formalin and malaria pigments.

Mercury pigment

This pigment is seen in tissues which have been fixed in mercury-containing fixatives, although it is rarely seen in tissue fixed in Heidenhain's Susa. Mercury pigment varies in its appearance but it is usually seen as a brown-black, extracellular crystal. Although usually seen as monorefringent, occasionally it is birefringent, particularly when formalin-fixed tissue has been secondarily fixed in formal mercury.

A little-known but unusual finding is that prolonged storage of stained sections which contain mercury pigment can bring about a change in the structure of the pigment. The pigment changes from crystalline to a globular form. The reason for this is unclear but it may be caused by interaction between the pigment and the mounting medium. Furthermore, the globular form exhibits a Maltese cross birefringence.

Treatment of sections with iodine solutions, such as Lugol's iodine, is the classical method of removing the pigment. Subsequent bleaching with a weak sodium thiosulfate (hypo) solution completes the treatment.

It is advisable *not* to remove mercury pigment with iodine solutions prior to staining with Gram's method. The effect is such that connective tissue will take up the crystal violet and then resist acetone color removal. Staining methods such as phosphotungstic acid hematoxylin may be impaired if 'hypo' is used before staining.

Chromic oxide

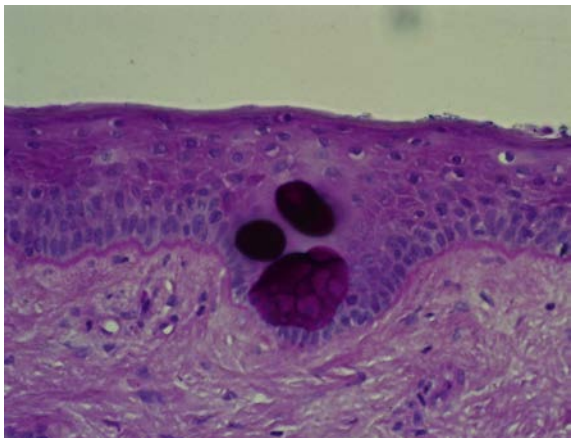
This pigment is rarely seen in tissue sections and is extremely difficult to produce intentionally. When seen, it presents as a fine yellow-brown particulate deposit in tissues, as a result of not washing tissues which have been fixed in chromic acid or dichromate-containing fixatives sufficiently in water. Subsequent treatment of tissues with graded alcohols, as used in tissue processors, may result in the reduction of chrome salts to the chromic oxides, which are insoluble in alcohol. The pigment is monorefringent and extracellular. It can be removed from sections by treatment with 1% acid alcohol.

Starch

This pigment is introduced by powder from the gloves of surgeons, nurses or pathologists. It is PAS and Gomori methenamine silver (GMS) positive and can easily be identified by its characteristic appearance. When polarized, it will produce a Maltese cross configuration (Fig. 14.19a and b).

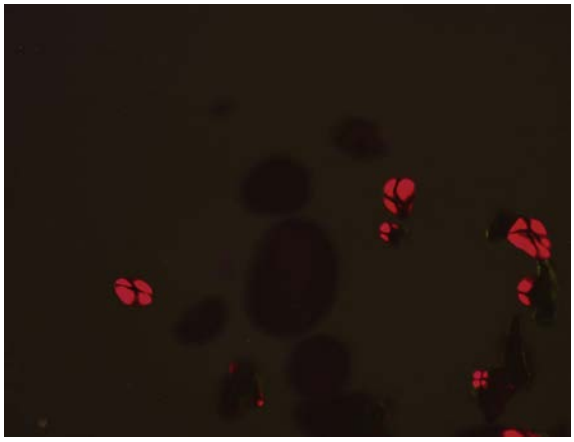
Exogenous pigments and minerals

Although often listed as being exogenous pigments, the majority of the following substances are, in fact, colorless. Some of these substances are inert and unreactive, while other materials can be visualized in tissue sections using various histochemical methods which are often capricious and unreliable. Most routine surgical pathology laboratories rarely see this type of material. Certain types of mineral gain access to the body by inhalation, ingestion or skin implantation, commonly as a result of industrial exposure. Some minerals, in the form of dye complexes, can be seen in the skin and adjacent lymph nodes as a result of tattooing. Occasionally mineral deposition may occur due to medication



a

Fig. 14.19a A section of skin stained with PAS showing positive staining of starch grains.



b

Fig. 14.19b A section of skin stained with PAS showing starch grains with the characteristic Maltese cross appearance when viewed under polarized light.

or wound dressing. Where there is a need to identify one of these substances, for example, with an industrial injury insurance claim, then use of the electron probe micro analyzer (EDAX) will prove to be the most reliable method. This specialized piece of equipment can usually be found in teaching and research laboratories.

The most common minerals seen in tissue sections are carbon, silica and asbestos. Other less common minerals which may be present in tissues are lead, beryllium, aluminum, mercury, silver and bismuth.

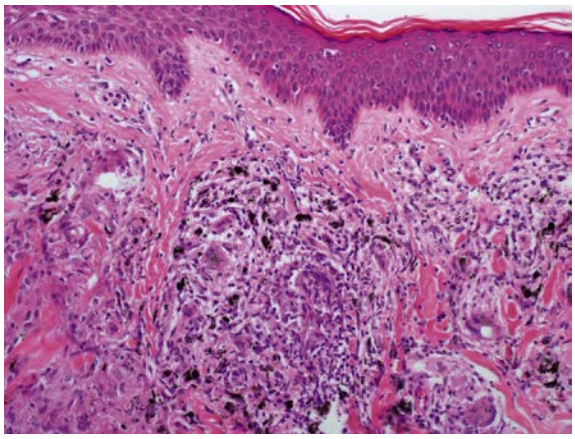


Fig. 14.20 A section of skin demonstrating exogenous tattoo pigment in a tattoo granuloma stained with hematoxylin and eosin.

Tattoo pigment

This is associated with skin and any adjacent lymphoid areas. If viewed using reflected light, the various colors of the dye pigments used to create the tattoo can be seen (Fig. 14.20).

Amalgam tattoo

Brown-black areas of pigmentation in the mouth may result from traumatic introduction of mercury and silver from dental amalgam during dental procedures. Histologically, brown granules are deposited in collagen, basement membranes, nerve sheath, blood vessel walls and elastic fibers. The pattern of distribution is similar to that seen in the skin in argyria.

Carbon

This exogenous substance is the most commonly seen mineral in tissues and is easily recognized in stained tissue sections. Commonly found in the lung (Fig. 14.21) and adjacent lymph nodes of urban dwellers, the main sources of this material are car exhausts and smoke from domestic and industrial pollution. Tobacco smokers inhale particulate carbon, and also give passers-by a small sample. Inhaled carbon particles generally are trapped by the thin film of mucus in the nose, pharynx, trachea and bronchi. A small amount can find the way into

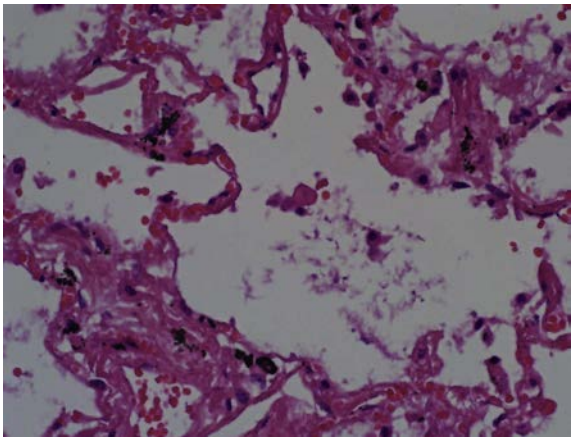


Fig. 14.21 A section of lung stained with hematoxylin and eosin, demonstrating carbon deposits (black) in and around the alveoli sacs.

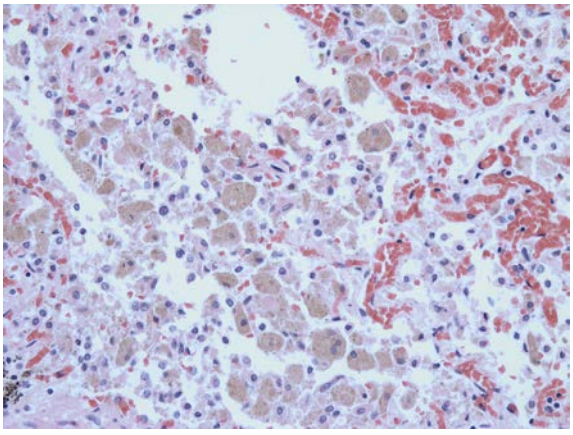


Fig. 14.22 Tobacco pigment (yellow-brown color) is seen within alveolar macrophages.

the alveoli of the lung, where the particulate matter is phagocytosed by alveolar macrophages. Some carbon particles will also find their way into the peribronchiolar lymphatics and lymph nodes draining the lungs.

Heavy black pigmentation of the lung (anthracosis) may be seen as a result of massive deposition of carbonaceous matter in coal workers. Indeed, the lungs can appear almost black. Whilst not all coal workers will develop lung disease, the lung disorder known as coal workers' pneumoconiosis is caused by the inhalation of silica, coal dust and many other particulates. The silica and other minerals are found in association with coal and other mined ores. Coal workers are also prone to cuts and abrasions whilst working, and coal 'tattoos' are fairly common in the skin.

The carbonaceous material is relatively inert and fails to be demonstrated with conventional histological stains and histochemical methods. The site and nature of the carbon deposits make identification relatively easy. In skin tissues it can be confused with melanin deposition, but treatment with bleaching agents will show carbon unaffected, whereas in the case of melanin the color will disappear.

Tobacco

Aside from various particulates and fibers inhaled in daily life as a result of urbanization and industrialization, it is clear that the social pastime of smoking organic, and other products, may leave some pigmented residue in the lung tissues. The best example is that of tobacco pigment, being seen as a golden-brown finely granular pigment. This is particularly concentrated in alveolar macrophages (Fig. 14.22) and may be seen in surgical as well as autopsy samples. It is also clear that smoking cannabis and some related products can produce similar pigment changes, although persons using these drugs often are tobacco smokers as well. There is no specific histological pigment/test required for confirmation of this pigment, beyond that of the material being negative in terms of the Perls' reaction and showing no staining to suggest lipofuscin or another endogenous pigment.

Silica

Silica, in the form of silicates, is associated with the majority of all mined ores because they are found in, or near, rocks which contain silica. Mine workers can inhale large quantities of silica, which can give rise to the disease silicosis. This disease may present as a progressive pulmonary fibrotic condition which gives rise to impaired lung capacity and in some cases extreme disability. Silicates are also abundant in stone and sand, and any industrial worker involved in grinding stone, sandblasting

or equivalent will be at risk from silicosis. Silica is unreactive, and is thus not demonstrated by histological stains and histochemical methods. It is weakly anisotropic (birefringent) when examined using polarized light, but the coexisting mica absorbed does show as refractile particulate matter. The histology of the scarring, nevertheless, is fairly characteristic.

Asbestos

Asbestos has been used for many years as a fire-resistant and insulating material. There are two groups of asbestos which cause pulmonary disease in humans: serpentine (curly fibers) and amphibole (straight/chain-like fibers). There is only one type of serpentine asbestos known as white asbestos (chrysotile) but there are many types of amphibole asbestos, the most common are blue asbestos (crocidolite) and brown asbestos (amosite) (Fig. 14.23). Perhaps the most dangerous type is crocidolite. The fibers are 5–100 µm long and only 0.25–0.5 µm in diameter, and can collect in the alveoli at the periphery of the lung. These fibers are anisotropic, but fail to show birefringence when they appear as an asbestos body because of the protein coat covering the fiber. The characteristic appearance of the asbestos body is as a beaded, yellow-brown, dumb-bell shape in lung sections. The proteinaceous coat contains hemosiderin and is positive with Perls' Prussian blue. Micro-incineration techniques can be used to demonstrate asbestos fibers as they withstand the high temperatures produced by the oven. In cases where asbestosis is suspected but no asbestos fibers or bodies are readily demonstrable, lung tissue can be digested with 40% sodium hydroxide or ashed. The resultant tissue sludge is then centrifuged and washed in water. Smears from the deposit are made and examined using polarized light or using transition electron microscopy +/− EDAX. This method has proved to be reliable when detecting/counting asbestos fibers and bodies. Alternatively, thick paraffin sections (20 µm) of lung tissue are mounted on glass slides coated with an adhesive. The sections are dewaxed and mounted unstained, then examined using polarized light.

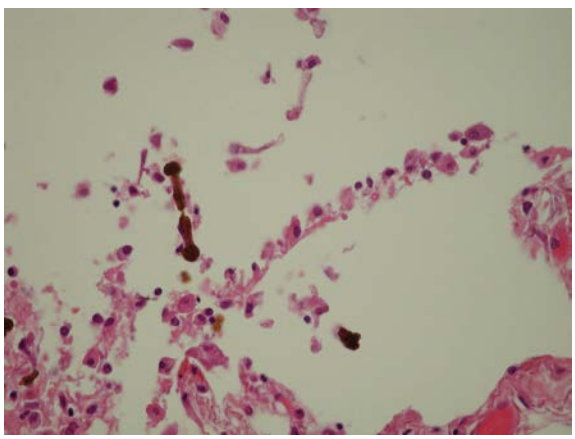


Fig. 14.23 H&E-stained section of lung alveoli demonstrating an amphibole fiber (ferruginous body) coated with hemosiderin pigment in a case of mesothelioma.

Lead

Environmental pollution due to lead has greatly reduced in recent decades. Lead pipes which carried much of the domestic water supply have been replaced by alternative materials. Lead in paint, batteries and gasoline has been reduced by the various manufacturers. Cases of lead poisoning are rare and are usually diagnosed biochemically using the serum from suspected cases. In chronic lead poisoning, excessive amounts can be deposited within many tissues, particularly bone and kidney tubules. For many years various methods have been used to demonstrate lead in tissue sections; the most popular method is the rhodizonate method (Lillie, 1954) (Fig. 14.24). Other methods for lead include the sulfide-silver of Timm (1958) and the unripened hematoxylin technique of Mallory and Parker (1939), although neither of these is specific for lead.

Rhodizonate method for lead salts (Lillie, 1954)

Fixation

Avoid the use of mercury-containing fixatives. Bones containing lead salts can be decalcified in 5–10% sulfuric acid containing 5–10% sodium sulfate. This procedure should convert lead deposits into insoluble lead sulfate.

Sections

Paraffin wax embedded.

Solutions

Rhodizonate solution

Sodium rhodizonate	100 mg
Distilled water	50 ml
Glacial acetic acid	0.5 ml

0.05% fast green FCF (42053) in 0.02% acetic acid

Method

1. Take sections to distilled water.
2. Place in rhodizonate solution for 1 hour.
3. Rinse well in distilled water.
4. Counterstain in 0.05% aqueous fast green in 0.2% acetic acid for 1 minute.
5. Rinse in three changes of distilled water.
6. Dehydrate, clear and mount in synthetic resin.

Results

Lead salts	black
Background	green

Notes

a. This method relies on any lead salts present forming a red chelated compound when treated with the chelating agent sodium rhodizonate.

b. This method can be performed using a microwave oven by heating the solution to 60–65°C and allowing the slides to remain in the heated solution for 5 minutes.

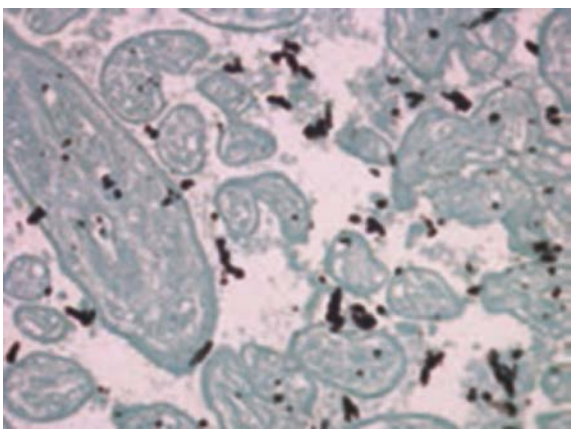


Fig. 14.24 A section of placenta which was treated with lead nitrate stained with Lillie's rhodizonate method for lead. Lead is stained black.

Beryllium and aluminum

The same methods are used to demonstrate both of these metals. It is therefore convenient to consider both together. Beryllium, used in the manufacture of fluorescent light tubes, gains access to the body by inhalation or traumatization of the skin. A foreign body granuloma is formed, often resembling the appearance of sarcoidosis. Conchoidal (shell-like) bodies can also be found, which are typical of, but not specific to beryllium. These bodies usually give a positive reaction with Perls' Prussian blue.

Aluminum is rarely seen in tissues, but gains access to the body in a similar way to beryllium. It can also be found in bone biopsies from patients with encephalopathy on regular hemodialysis for chronic renal failure. Prolonged dialysis can cause osteodystrophy in the present era. It can develop insidiously and may present with a non-specific ache. The most severe pain occurs with osteomalacia, particularly when it is associated with aluminum deposition (Brenner, 2004). Beryllium and aluminum can both be demonstrated by solochrome azurine which forms a deep blue chelate. Aluminum is also positive with the fluorescent Morin method (Pearse, 1985), together with other minerals such as calcium, barium and zirconium. Naphthochrome green can also be used to demonstrate beryllium and aluminum, but is less specific than the solochrome azurine method as other metallic dye lakes can be formed.

Solochrome azurine method for beryllium and aluminum (Pearse, 1957)

Fixation

Not critical.

Sections

Paraffin wax or frozen.

Solutions

a. 0.2% solochrome azurine (Pure blue B)

b. 0.2% solochrome azurine in normal sodium hydroxide

Method

1. Take two test sections to distilled water.
2. Stain one section in solution **a** and one in solution **b** for 20 minutes.

3. Wash in distilled water.
4. Lightly counterstain in 0.5% aqueous neutral red or 0.1% aqueous nuclear fast red for 5 minutes.
5. Wash in distilled water and mount in synthetic resin.

Results

Solution **a**: aluminum and beryllium	blue
Solution **b**: beryllium only	blue-black
Nuclei	red

Notes

a. This method is reliable and will give consistent results.
b. Control sections should always be used if readily available.
c. Aluminum will fail to react at an alkaline pH.
d. A modification of this method applicable to resin sections of undecalcified bone is given in Chapter 16 of the previous edition of this text.

Aluminon method for aluminum
(Lillie & Fullmer, 1976)

Sections

Undecalcified glycol methacrylate or paraffin sections cut at 5 µm.

Solutions

pH buffer 5.2
Dissolve 40 g ammonium acetate and 28 g ammonium chloride in 210 ml distilled water. Add 27 ml 6 M (50% conc HCl) hydrochloric acid. Adjust to pH 5.2 with hydrochloric acid or 28% ammonium hydroxide. Store in a refrigerator at 3–6°C.

Aluminon solution
Dissolve 0.8 g aluminon (aurine tricarboxylic acid) in 40 ml pH buffer 5.2 with the aid of heat to 80–85°C. Prepare just before use.

Decolorizing solution
To 22 ml pH buffer 5.2 add 8 ml 1.6 M ammonium carbonate which consists of 15.4 g ammonium carbonate in distilled water to make a total of 100 ml.

Fast green counterstain
Dissolve 0.05 g fast green FCF (CI 42053) in 100 ml 0.2% acetic acid.

Method

1. Take sections to distilled water.
2. Pour freshly prepared aluminon solution which is heated to 80–85°C in a plastic Coplin jar. Place slides in this solution.
3. Place Coplin jar in a 600 W microwave oven and microwave at 120 W for 30 seconds (see Notes below). Allow the slides to remain in the solution for 10 minutes.
4. Rinse in three changes of distilled water.
5. Place in freshly prepared decolorizing solution for 5 seconds.
6. Rinse in three changes of distilled water.
7. Counterstain with fast green solution for 3 minutes.
8. Rinse in three changes of distilled water.
9. Stand slides on end and allow slides to air dry.
10. Dip in xylene and mount with synthetic resin.

Results

Aluminum	red
Background	green

Notes

a. Currently available microwave ovens have a maximum wattage of 900–1500 W. Therefore, when using a microwave oven greater than 600 W, the time of exposure to microwaves should be reduced proportionally in Step 3.
b. Another counterstain which may be used in this method is methylene blue as it contrasts well with the red positive stain (Fig. 14.25), like the fast green.

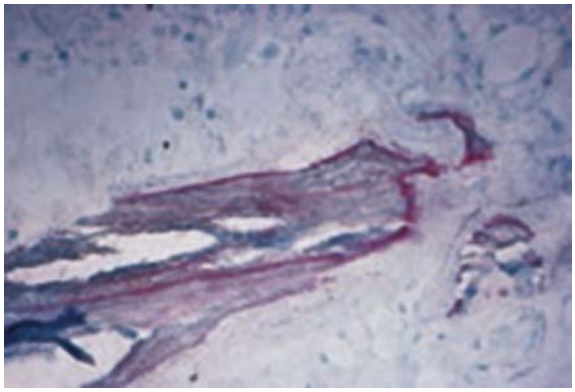


Fig. 14.25 An undecalcified plastic section of bone stained with Lillie's aluminon method for aluminum. Aluminum is stained red.

Silver

Silver is occasionally found in the skin of silver workers as a result of industrial exposure. It is now more commonly seen as a localized change in the mouth (amalgam tattoo, see above) or in association

with silver earrings in ineptly pierced lobes. The resultant permanent blue-gray pigmentation is called argyria and is most marked in those areas exposed to sunlight. In unstained and H&E-stained sections the silver appears as fine dark brown or black granules, particularly in basement membranes and sweat glands. The method of Okamoto and Utamura (1938), a metal chelating method which utilizes dimethylaminobenzylidene-rhodanine, will demonstrate silver. The method tends to be capricious and may give rise to the diffusion of chelate complex. The best results are obtained using frozen sections.

Rhodanine method for silver (Okamoto & Utamura, 1938)

Fixation

Not critical but avoid the use of mercury-containing fixatives.

Sections

Paraffin wax; frozen (see Note a below).

Incubating solution

P-Dimethylaminobenzylidene-rhodanine (saturated solution in 90%)	3.5 ml
M nitric acid	3 ml
Distilled water	93.5 ml

Method

1. Take paraffin wax sections to distilled water.
2. Incubate sections in rhodanine solution at 37°C for 24 hours.
3. Wash well in distilled water.
4. Mount in glycerin jelly.

Results

Silver deposits	red-brown

Notes

a. Better results are obtained on free-floating frozen sections using a 0.2% rhodanine solution in 0.1% nitric acid for 2 hours at 37°C.

b. The reaction product is not completely insoluble and some diffusion occurs, so sections should be examined immediately. Long incubation times will give poor localization.

c. The exogenous pigments, particularly of the elements, can also be analyzed for tissue density by mass spectrometry.

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15 Amyloid

Janet A. Gilbertson

Introduction

History

Amyloidosis is a disorder of protein folding, in which normally soluble proteins misfold and aggregate in a characteristic highly ordered fashion, and are deposited in the extracellular space as insoluble fibrils (or filaments). These interstitial fibrillar protein deposits (generically known as amyloid) may form anything from microscopic plaques to confluent masses. Amyloid can progressively replace the parenchyma of affected organs, which may become significantly enlarged, damaging the tissue structure and function. This eventually leads to organ failure, often resulting in death.

The term amyloid means 'starch-like' and was first used to describe the starchy cellulose material found in plants, giving a deep blue/violet color with iodine. This reaction was adapted by Virchow (1853, 1854) who found that corpora amylacea of the brain had the same tinctorial properties as starch. He went on to investigate tissues containing 'real' amyloid, which he found also gave the same coloration. To date, amyloid continues to be defined and identified by its characteristic histological staining reactions.

The first molecular investigations began in the mid-19th century with the observation at autopsy that the cut surface of amyloid-affected organs may show a 'waxy' texture, often described by early pathologists as 'waxy degeneration' or 'lardaceous disease' (von Rokitansky, 1842). Throughout this period this appearance was a frequent post-mortem finding in patients suffering from chronic inflammatory diseases such as tuberculosis (TB), although amyloidosis itself was rarely diagnosed during the patient's lifetime.

At that time the exact composition of amyloid was unknown, other than it had a proteinaceous/albuminous nature with a high nitrogen content. As techniques improved during the twentieth century, a consensus was made that amyloid was predominately composed of proteins with 1–5% mucopolysaccharide present.

With the development of the textile industry in the 1880s, dyes became readily available and used for many applications other than textiles, including histological techniques. Bennhold (1922) used the paper dye Crystal violet and the cotton dye Congo red for the demonstration of amyloid. Neubert (1925) noted the dichroic effect of Congo red stained-amyloid when viewed under polarized light. Subsequently, in 1927, the optical activity of Congo red-stained amyloid giving the unique 'apple-green birefringence' was described by Divry & Florkin. This phrase is still in use today to describe the birefringence and dichroism that amyloid displays when viewed under crossed polarized light, although Howie & Brewer (2009) suggested that the correct phrase should be of 'anomalous colors', describing all the colors amyloid stained with Congo red can produce. With the advent of electron microscopy in the 1950s it was observed that amyloid had a unique fibrillary ultrastructure independent of anatomical site; quite different to any other ultrastructural fibrils described previously. In 1968, Eanes and Glenner used X-ray diffraction to reveal that the protein within amyloid fibrils was arranged in an anti-parallel β-pleated sheet (Fig. 15.1).

Since then it has been shown that all amyloid fibrils share a common β-core structure with polypeptide chains running perpendicular to the fibril long axis, regardless of the particular protein from which they are formed (Sunde et al., 1997).

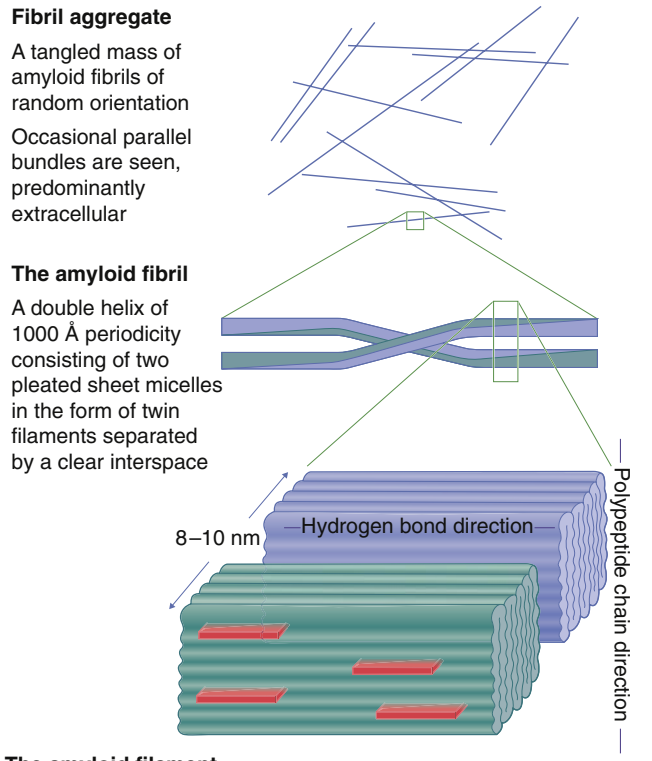

Fig. 15.1 Schematic diagram of amyloid structure.

Composition

Following the development of techniques enabling amyloid fibril isolation (Pras et al., 1968; Glenner et al., 1972) it was confirmed that the bulk of amyloid deposits were composed of protein. They also contain up to 15% of a non-fibrillary glycoprotein known as amyloid P component (AP), derived from, and identical to the normal circulating plasma protein serum amyloid P (SAP). SAP is a calcium-dependent, ligand-binding protein which also forms a normal component of basement membranes and elastic fibers, and may have a function related to its binding to glycosaminoglycans (GAGs), fibronectin and other cellular components (Pepys & Baltz, 1983). SAP belongs to the pentraxin family, and becomes specifically and highly concentrated in amyloid deposits of all types. This binding specificity to amyloid fibrils is the basis for radiolabeled SAP scintigraphy in patients with amyloidosis, a diagnostic technique which also enables quantitative monitoring of amyloid deposits (Hawkins et al., 1990). The generic SAP ligand on amyloid fibrils remains uncharacterized (Pepys, 1992).

The composition of the GAGs includes various sulfates, heparan, chondroitin and dermatan, which may be involved in amyloidogenesis. The presence of the carbohydrate moieties of GAGs provides a possible explanation for the staining reaction in some of the histological methods used for amyloid detection.

Ultrastructure

Electron microscopy (EM) played an important role in the identification of the composition of amyloid, showing its unique fibrillary arrangement. To this day, EM is one of the methods for identifying

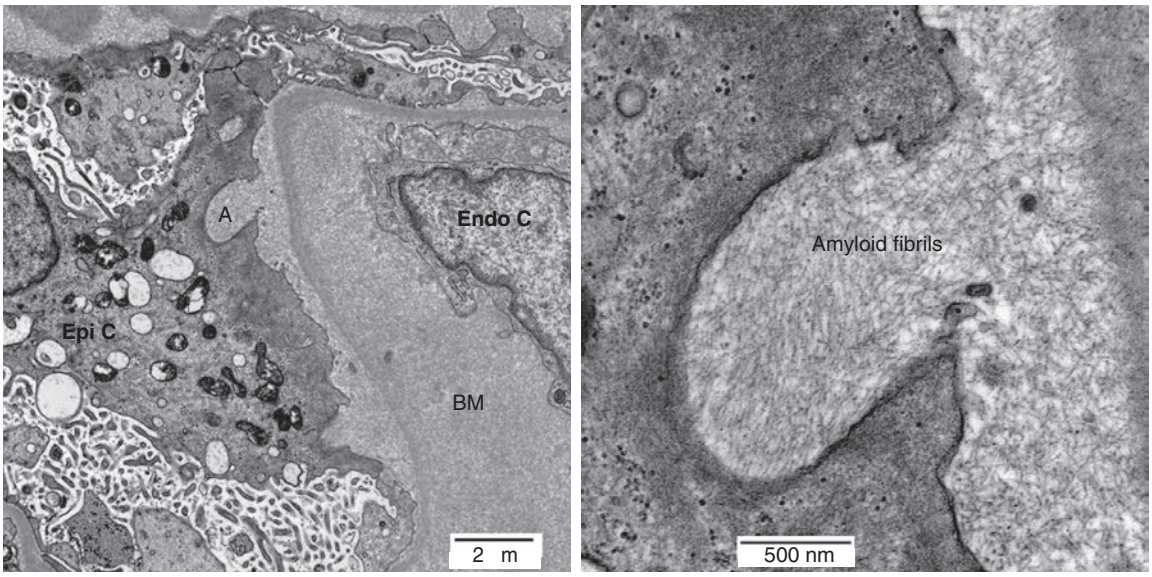

Fig. 15.2 (a) Electron micrograph of kidney from a case of amyloidosis with renal involvement showing amyloid (A), epithelial cytoplasm (Epi C), basement membrane (BM) and an endothelial cell (Endo C). (b) A higher magnification of Fig. 15.2a where amyloid fibrils can be clearly seen.

amyloid. Amyloid deposits appear as masses of extracellular, non-branched filaments, usually in a random orientation (though occasionally in parallel arrays of a few fibrils). Each fibril consists of two electron-dense filaments 2.5–3.5 nm in diameter, separated by a 2.5 nm space, giving a total diameter of 8–10 nm with variable length of up to several μm (Cohen & Calkins, 1959; Glenner, 1981) (Fig. 15.2a, b).

Classification and nomenclature

There have been many attempts to identify and classify amyloid proteins in order to collate the endless variety of clinical manifestations, histopathological appearances and associated pathology. Until 1980, traditionally the main classification used for amyloid was that of Reimann et al. (1935), who divided amyloid types into four categories:

1. Primary amyloid occurring spontaneously in the absence of an apparent predisposing illness. It affects organs and tissues such as heart, muscle, skin and tongue.
2. Secondary amyloid occurring in patients with chronic infective diseases, e.g. syphilis and TB. Later, inflammatory diseases such as rheumatoid arthritis were also included in this group.
3. Tumor-associated amyloid.
4. Myeloma-associated amyloid.

As more and more amyloid-forming proteins were identified, this classification became obsolete. In 1974, a committee was organized at the international symposium on amyloidosis in Finland to oversee the nomenclature, while in 1990, Husby et al. proposed guidelines for a better classification based on the identity of the amyloid fibril protein. This classification was adopted by the World Health Organization-International Union of Immununological Societies (WHO-IUIS). Today, this forms the currently accepted classification of amyloid. The International Society of Amyloidosis (ISA) meets biennially and at each symposium reviews any new types and possible new nomenclature.

At present, there are 36 different proteins which have been accepted as major amyloid-fibril proteins (Table 15.1). Amyloidosis nomenclature uses the letter A to designate amyloid, followed by an abbreviation of the name of the fibril protein. For example, immunoglobulin light chain amyloid protein is

Table 15.1 Unrelated amyloid-forming proteins

Abbreviation	Protein precursor	Dominant tissues affected	Amyloid type
AA*	Serum amyloid A protein	Spleen, gut	Reactive systemic AA amyloidosis
AL (κ & λ)*	Immunoglobulin light chain	Any tissue except the brain parenchyma	Systemic AL amyloidosis or localized AL amyloidosis
AH*	Immunoglobulin heavy chain	Renal	Systemic AL amyloidosis or localized AL amyloidosis
ATTR*	Variant transthyretin (pre-albumin)	Peripheral and/or autonomic nerves, cardiac, gastro-intestinal	Familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC)
ATTRwt*	Wild-type transthyretin (prealbumin)	Cardiac	TTR not hereditary
AApoAI*	Variant apolipoprotein AI	Kidney, liver	Hereditary systemic amyloidosis
AApoAII*	Apolipoprotein AII	Kidney, liver	Hereditary
AApoAIV*	Apolipoprotein AIV	Kidney, liver	Sporadic aging amyloidosis
AApoCII	Apolipoprotein CII	Kidney	Hereditary
AApoCIII	Apolipoprotein CIII	Kidney	Hereditary
AGel*	Gelsolin	Kidney	Hereditary
ACys*	Cystatin C	Brain	Hereditary
Aβ	Amyloid β-protein precursor	Brain	Alzheimer's disease, Down's syndrome, cerebral amyloid angiopathy
$A\beta_2M$*	β_2-Microglobulin	Kidney, prostate	Dialysis-associated amyloid, corpora amylacea
APrP	Prion protein	Brain	Creutzfeldt-Jacob disease (CJD), prion disease
ACal	Calcitonin	Thyroid	Medullary carcinoma of thyroid-associated disease
AANF	Atrial natriuretic factor	Heart	Cardiac amyloid
AIAPP	Islet amyloid polypeptide (Amylin)	Pancreas	Type II diabetes amyloid, insulinoma
AFib*	Fibrinogen A α-chain	Kidney	Hereditary
ALys*	Lysozyme	Kidney	Hereditary
APro	Prolactin	Pituitary	Aging amyloid
AIns	Insulin	Skin / soft tissue	Amyloid derived from insulin at injection sites
ABri	ABriPP	Brain	Familial dementia (British)
ADan	A Dan PP The product of the same gene ABri	Brain	Familial dementia (Danish)
AMed	Lactadherin	Arteries	Aging amyloid in arteries
ALac	Lactoferrin	Cornea	Corneal amyloidosis
AKer	Kerato-epitheliun	Eyes	Familial corneal amyloidosis

Table 15.1 Unrelated amyloid-forming proteins—cont'd

Abbreviation	Protein precursor	Dominant tissues affected	Amyloid type
ALect2*	Leukocyte common antigen 2	Kidney, liver	LECT 2 amyloidosis
AGal7	Galectin 7	Skin	Localized
ACor	Corneodesmosin	Cornified epithelia, hair follicles	Localized
AOAPP	Odontogenic ameloblast-associated protein	Odontogenic tumors	Localized
ASem1	Semnogelin 1	Seminal gland	Localized
AEnf	Enfuritide	Skin, soft tissue	Iatrogenic, injection sites
ASpc	Lung surfactant protein	Lung	Localized
AαSyn	α-Synuclein	Central nervous system	Acquired
ATau	Tau	Central nervous system	Acquired

*One of the 14 forms of amyloid proteins which can give rise to systemic disease.

called AL (A + immunoglobulin light chain); this can be further added to by kappa (κ) or lambda (λ) subtype (e.g. ALλ type).

Pathogenesis

The processes which cause proteins to become involved in amyloid formation, i.e. the conversion from normal functioning proteins into inert amyloid deposits, is the focus of much research. There is little in common between many different types of proteins involved (Merlini & Bellotti, 2003). Many protein misfolding and amyloid aggregation diseases are strongly associated with aging, and diseases such as Alzheimer's and Parkinson's disease are becoming among the most common debilitating medical conditions throughout the world today. Whilst the close association between the appearance of amyloid deposits and the onset of pathological events is well described, the specific mechanisms underlying these events is still not understood (Knowles et al., 2014). In certain amyloid types, the fibrils contain only a limited cleaved portion of the amyloid protein precursor, as is the case in Alzheimer's disease.

Some amyloid fibril precursor proteins are rich in β-pleated sheet conformation in their native form, whereas others e.g. the prion protein, contain no β-pleated sheet and it is the de novo formation of β-pleated sheets which represent a fundamental pathological event in prion diseases. In some types of amyloidosis the whole precursor protein may be involved, or there may be proteolysis of the precursor protein with liberation of a smaller amyloidogenic fragment, as with AβPP. There could also be a mixture of two. In transthyretin (TTR) amyloidosis, the circulating protein is a tetramer, and a vital pathological event appears to be release of the monomer.

Amyloidosis is now considered to belong to the category of conformational diseases. The pathological protein aggregation reflects, at least in part, limited stability of the normal physiological conformation, with a propensity to adopt an alternative pathologic conformation. It has been proposed that such a grouping helps to provide an understanding of the etiology of these diseases. This opens the prospect for common approaches to therapeutic stratagems in a similar way that recognition of bacteria as the causative agents of many infections gave rise to the use of antibiotics being useful in such conditions, or of steroid therapy being of potential use for all inflammatory disorders (Carrell & Lomas, 1997; Carrell & Gooptu, 1998). In this context, it is interesting to note the development of 'designer' peptides which bind to Aβ and to prion proteins with the aim of preventing, and even reversing the conformational changes responsible for these respective disease processes (Soto et al., 2000). This concept is becoming increasingly accepted, and it is becoming

Table 15.2 Protein conformation diseases

Conditions	Affected proteins	Associated diseases
Amyloidosis	36 known in humans (Table 15.1)	
Serpinopathies	α_1-Antitrypsin neuroserpin	α_1-Antitrypsin storage disease
Hemoglobinopathies	Hemoglobulin	Sickle cell anemia Drug and aging induced inclusion body hemolysis
Lewy body diseases	α-Synuclein	Parkinson's disease
Neuronal inclusion bodies	Tau	Alzheimer's disease Pick's disease Progressive supranuclear palsy
	Superoxide dismutase	Motor neuron disease
	Ferritin	Familial neurodegenerative disorder
Hirano bodies	Actin	Alzheimer's disease
Polyglutamine repeats	Huntingtin	Huntington's disease
	Ataxin	Spinocerebellar ataxias
	Androgen receptor	Spinomuscular atrophies
Prion diseases	Prion protein	CJD Variant CJD Gerstmann-Straussler-Scheinker disease Kuru Fatal familial insomnia Japanese cerebral amyloid angiopathy (CAA)

evident that amyloid is but one, albeit definable, subgroup within a larger group of misfolded or altered protein deposits which are associated with human disease (Table 15.2).

Amyloidosis

In AL amyloidosis, previously known as 'primary amyloidosis', monoclonal immunoglobulin light chains, produced by acquired clonal plasma cell or other B-cell dyscrasias, form amyloid deposits widely throughout the tissues. These can be either of kappa (κ) or lambda (λ) isotypes. Systemic AL amyloidosis is the most common form of clinical amyloid disease in developed countries and causes the most fatalities. In systemic amyloidosis, deposits can be present in any or all of the viscera, connective tissues and blood vessel walls, although intracerebral amyloid deposits are never found (Pepys, 2006).

AL amyloidosis can also be localized, i.e. restricted to a particular organ or tissue, and usually has a benign prognosis. In the skin, the deposits cause benign lumps which can be excised or left untreated. Localized amyloid deposits are also common in the bladder and pulmonary tissue where they can cause obstruction, leading to complications.

There are five possible types of heavy chain amyloidosis (AH); G, A, D, E and M – these are rare.

AA amyloidosis, previously known as 'secondary amyloidosis', is a complication of a prolonged chronic infection and/or inflammatory condition. AA

amyloidosis is consequent on a longstanding acute phase response, in which production of serum amyloid A protein (SAA) is greatly increased. SAA is an apolipoprotein produced in the liver. It is an acute phase protein which is synthesized at increased levels in patients with diseases such as rheumatoid arthritis, TB, Crohn's disease, familial Mediterranean fever (FMF) and other hereditary periodic fevers.

Due to therapeutic developments in many inflammatory diseases over the last 20 years, the incidence of AA amyloidosis has greatly decreased. However, there are now cases associated with intravenous drug abuse and HIV infection. In approximately 6% of cases of AA amyloidosis, the underlying inflammatory disorder cannot be characterized (Lachmann et al., 2007). Abnormally increased production of SAA over a long period is a prerequisite for development of AA amyloidosis.

There are various types of hereditary systemic amyloidosis which involve many different organ systems. They are difficult to treat and often fatal. This group of disorders are dominantly inherited and rare, but there are various clusters around the world. One of the most common types is due to point mutations in the TTR gene; there are approximately 10,000 affected individuals worldwide. With more than 100 amyloidogenic mutations in the TTR gene, the major features of hereditary TTR amyloidosis include severe, ultimately fatal peripheral and/or autonomic neuropathy (familial amyloid polyneuropathy, FAP), and/or cardiac amyloidosis. Hereditary amyloidosis is also associated with mutations in the genes encoding apolipoproteins AI and AII, fibrinogen α-chain, gelsolin, lysozyme, cystatin C and β-protein.

Although all forms of hereditary amyloidosis are inherited dominantly, the penetrance and expressivity are remarkably variable. Thus there may be marked differences in age of onset, amyloid deposition and clinical presentation, not only between families but also within families with the same mutation. In contrast to AA amyloidosis (in which the concentration of the amyloid fibril protein SAA is raised but of normal structure), AL and hereditary amyloidosis are associated with proteins which have abnormal structure conferring an inherent propensity to undergo aberrant folding rich in β-pleated sheets to form amyloid fibrils.

Leukocyte cell-derived chemotactin 2 (ALECT2) amyloidosis was discovered by Benson et al. in 2008 whilst characterizing amyloid co-existing in a clear cell carcinoma nephrectomy specimen. When studying proteins with leukocyte chemotactic activity Yamagoe et al. (1996) found LECT2 to stimulate chondrocytes and osteoblasts. However, the pathogenesis of LECT2 amyloid remains to be understood. Whilst there is no evidence that ALECT2 is an inherited condition, the disease has a strong ethnic bias with most patients being Hispanic, Mexican, Punjabi and Native American (Larsen et al., 2010). ALECT2 amyloid deposits predominantly affect the liver and kidneys.

Wild type transthyretin (ATTRwt) amyloidosis, previously referred to as senile systemic amyloidosis, is not hereditary, the precursor protein being normal plasma transthyretin. ATTRwt amyloid deposits can be found in the myocardium of up to approximately 25% of elderly subjects, with men being more affected than women. However, cardiac deposition sufficient to cause clinical disease is apparently rare, or perhaps under-diagnosed. Deposition of wild type transthyretin also occurs in other anatomical sites, including the prostate, bladder and blood vessel walls, but only occasionally with clinical consequences.

Other diseases in which amyloid occurs

As outlined by Pepys in 2006, amyloid is a histological feature of Alzheimer's disease and type 2 diabetes mellitus but, unlike systemic amyloidosis, it is not known whether the amyloid causes these diseases. In Alzheimer's there is an abundance of intracerebral amyloid deposits composed of β-protein, but there is poor correlation between the quantity of amyloid and the cognitive impairment. However, mutations which result in abundant deposition of β-protein as amyloid may result in early-onset Alzheimer's disease.

In patients with type 2 diabetes, amyloid is frequently found in the pancreatic islets of Langerhans (IAPP), though this is not universal in all islets or in all patients with type 2 diabetes. As well as this, in diabetic patients amyloid can also be found at the site of insulin injection; as a result of the injected insulin (AIns), this causes a localized cutaneous lump. Insulin is known to be able to convert into a fibrillary form in vitro when subjected to certain

physical or chemical stimuli such as heat or acidity (Lonsdale-Eccles et al., 2009).

It is also frequently cited that transmissible spongiform encephalopathy (TSE) is an example of amyloidosis, although amyloid deposits are not necessarily present in the brains of patients with the disease, nor in cows with bovine spongiform encephalopathy (BSE).

There are other protein misfolding disorders characterized by abnormal aggregates of proteins which are incorrectly described as amyloid-related, e.g. Parkinson's disease associated with Lewy bodies and the polyglutamine repeats which cause Huntington's disease.

Another protein deposition disease, often confused with amyloid, is light chain deposition disease (LCDD), which may be mistaken histologically due its accumulation in the extracellular space. However, LCDD deposits lack the affinity for Congo red stain and do not produce the characteristic green birefringence of amyloid under cross-polarized light. Under EM, LCDD deposits are granular, which aids the distinction (Gibbs & Hawkins, 2011).

Corpora amylacea (CA) or 'false amyloid' is not a disease as such but small benign masses which are composed of bundles of hyaline fibrils having the same Congo red staining properties as amyloid. CA is found as luminal concretions in prostate, lung and uterus.

Diagnosis

There is now an increased awareness of amyloidosis and more patients are being diagnosed and referred to central amyloid management units such as the National Amyloidosis Centre (NAC) in the UK.

However, some patients are still overlooked. The diagnosis requires the presence of amyloid in a tissue. The gold standard technique is Congo red histology, although EM may aid diagnosis. Biopsies are usually taken to investigate organ dysfunction e.g. of the kidneys in nephrotic patients, or of sural nerves in familial polyneuropathies. Amyloid is present in up to 90% of rectal and/or subcutaneous fat biopsies in systemic AA or AL amyloidosis; rectal biopsies or fine needle aspirates of subcutaneous tissue used to be the main method of screening (Westermark & Stenkvist, 1973; Pepys, 1992). Techniques have now improved greatly and cardiac biopsies, after a suggestive echocardiogram, are becoming more popular. It must be noted that a negative biopsy does not exclude the possibility of amyloidosis. In rectal biopsies, amyloid is usually found in the walls of submucosal vessels, so if the full thickness of the muscularis is not obtained the deposits will go undetected.

The use of SAP scintigraphy allows in vivo diagnosis as well as the monitoring of progression and regression of the amyloid deposits with treatment (Hawkins et al., 1993; Hawkins, 1994). Unfortunately, SAP scintigraphy is unable to visualize amyloid within cardiac tissue because the heart is a moving organ. The bone scanning method DPD scintigraphy (^{99m}Tc-3,3-diphosphono-1,2-propanodicarboxylic acid) was serendipitously discovered to have high affinity for cardiac ATTR amyloid, and is routinely performed as a method for imaging cardiac involvement in this type of amyloidosis (Hutt et al., 2014).

Differentiation between different amyloid types

With the recognition that different proteins form amyloid and are associated with different clinical syndromes it became necessary to identify particular fibril types histologically. As the treatment of amyloidosis is entirely type-specific, the correct identification of the fibril type is indispensable in clinical practice.

Methods of section pretreatment using trypsin or potassium permanganate before Congo red staining were devised (Wright et al., 1976, 1977). After such pretreatment some amyloids lose their affinity for Congo red, most notably AA amyloid, whereas AL amyloid is resistant. These methods were always equivocal in practice and have been rendered obsolete by the use of immunohistochemistry and other techniques to identify the particular amyloid fibril protein specifically and reliably.

It is vitally important to discriminate between AA, AL and hereditary amyloidosis, as their treatments are entirely different. Therapy for patients with AA amyloidosis involves measures to reduce SAA production by treating the cause of the underlying

inflammation. AL treatment is aimed at ablating the B-cell clone responsible for the amyloidogenic free light chain production using cytotoxic drugs. Hereditary amyloidosis treatment may ultimately result in organ transplantation.

The tools available today to differentiate amyloid type include direct assessment of the fibril type by immunohistochemistry, proteomics and, occasionally, fibril sequencing. Indirect investigations aimed at identifying the disorders associated with amyloidosis include searches for monoclonal immunoglobulins using conventional electrophoresis, immunofixation assays of serum and urine, the serum free light chain assay, serum assays for SAA, and sequencing of genes known to be associated with hereditary amyloidosis.

Demonstration

In hematoxylin and eosin (H&E) stained sections amyloid appears as an amorphous, eosinophilic, extracellular, faintly refractive substance which sometimes displays green birefringence under polarized light. However, it should be noted that collagen also has this appearance under polarized light in H&E-stained sections. Amyloid can also be weakly birefringent using a powerful light source when stained with periodic acid-Schiff. Whilst large deposits of amyloid can be identified in H&E-stained sections, small deposits, e.g. in vessels and rectal or bone marrow samples may be missed.

Congo red was developed as the first direct cotton dye in 1884, and has been 're-invented' many times in the search for an optimal method for the detection of amyloid (Puchtler et al., 1964). As with most histopathological methods, they are often performed on tissues which have been formalin fixed and processed into paraffin wax. Samples left standing in fixative for long periods of time may cause stains to be less sensitive and intense. Control sections must be used in all staining methods, and to demonstrate amyloid they should be cut when needed since they can lose reactivity if stored for longer than a year.

Congo red

The molecular formula for Congo red is $C_{32}H_{22}N_6Na_2O_6S_2$ (Fig. 15.3). It is a symmetrical

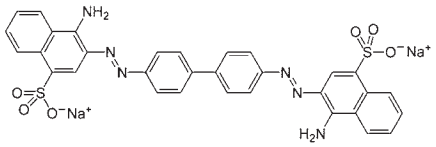

Fig. 15.3 Chemical structure of Congo red dye.

sulphonated azo dye containing a hydrophobic center with two phenyl groups bound by a diphenyl bond to give a linear molecule which is largely hydrophobic (Turnell & Finch, 1992). Congo red is also a fluorescent dye (Puchtler & Sweat, 1965), although not specific for amyloid. Two factors are important to the Congo red-amyloid reaction; the linearity of the dye molecule and the β-pleated sheet configuration. If the spatial configuration of either is altered, even though the chemical groupings are left intact, the reaction fails. Furthermore, the Congo red mediated positive birefringence of amyloid implies that the dye molecules are arranged in a parallel fashion (Romhányi, 1971). Recent work confirms the long-held belief that the Congo red molecule intercalates between two protein moieties at the interface between two adjacent antiparallel β-pleated sheets by disrupting the hydrogen bonds which are responsible for maintaining the β-sheet polymer, yet allowing maintenance of the integrity of the structure by the formation of new hydrogen bonds between protein and dye (Carter & Chou, 1998).

Since its introduction as a histological stain by Bennhold (1922), Congo red staining of amyloid producing green birefringence when viewed under polarized light has become the diagnostic gold standard for this disorder. Although it was many years before the exact staining mechanism was understood, it is now well established that staining of amyloid by Congo red is due to hydrogen bonding between the Congo red dye and the β-pleated sheet in a highly oriented linear and parallel manner on the amyloid fibrils (Glenner, 1980). Any tissue component which binds Congo red in a linear way also exhibits green birefringence in polarized light; dense collagen fibers also bind Congo red dye in this fashion, and various other formalin-fixed tissues can be stained. By using an alkaline Congo red method this phenomenon is reduced (Puchtler et al., 1962). However, Romhányi

used 1% aqueous Congo red and claimed that if the tissue sections were mounted in gum arabic this problem is overcome. Bély & Makovitzky (2006) adapted Romhányi's original method, using a long deparaffinization step of up to 5 days, together with a longer incubation in Congo red. This technique has shown that the amyloid has a stronger affinity to Congo red and therefore can be seen as more sensitive and selective. Many different tissue structures will also stain with 1% aqueous Congo red, and so it must be used under strict conditions using known amyloid-positive sections as controls.

The specificity of Congo red staining of amyloid can also be increased by using an alcoholic method combined with high ion strength and high pH. The Puchtler method (1962) combines all these aspects, giving a superior technique to demonstrate amyloid and green birefringence under polarized light.

A recent comparison of several Congo red staining methods made during a run of the UK NEQAS histology external quality control scheme found that Highman's method (1946) gave the highest scores. At the NAC a significant percentage of biopsies which are referred each year have been reported incorrectly, with both false positives (5%) and false negatives (8%) (Gilbertson et al., 2015a). This can have serious repercussions for patients' treatment. Consequently, the NAC recommends the Puchtler method since the lack of a differentiation step means there is less intervention by the operator. It should be noted that false positives and false negatives can be caused by other factors even in this technique, e.g. if the correct section thickness is not used.

Alkaline Congo red technique (Puchtler et al., 1962)

The method obviates the need for a differentiation step by the inclusion of a high concentration of sodium chloride. This reduces background electrochemical staining whilst enhancing hydrogen bonding of Congo red to amyloid, resulting in a progressive and highly selective technique. The solutions should be discarded after 1 month. Ready to use commercial reagents and kits are available with a shelf life of up to 12 months.

Fixation

Not critical.

Stock solutions

1% aqueous sodium hydroxide

Stock solution a

Saturated sodium chloride in 80% ethanol.

Stock solution b

Saturated Congo red in 80% ethanol saturated with sodium chloride.

Leave to stand overnight before use.

Working solutions

A. To 100 ml of stock solution **a** add 1 ml of 1% aqueous sodium hydroxide and filter.

B. To 100 ml of stock solution **b** add 1 ml of 1% aqueous sodium hydroxide and filter.

Prepare just before use.

Method

1. Take sections to water, removing pigment where necessary.
2. Stain nuclei in alcoholic alum hematoxylin (e.g. Mayer's) and blue.
3. Immerse in alkaline sodium chloride solution for 20 minutes (working solution **A**).
4. Drain briefly, it is important NOT to blot.
5. Transfer directly to the alkaline Congo red solution for 20 minutes (working solution **B**).
6. Rinse briefly in alcohol, clear and mount.

Results (Fig. 15.4)

Amyloid	red
Elastic and collagen fibers, eosinophil granules	pink
Nuclei	blue

Note

Although the stock solutions are stable for a couple of months, the working solutions do not keep and should be used within 20 minutes of preparation.

Highman's Congo red technique (Highman, 1946)

This simple method has found wide application. The solutions are relatively stable and the method affords a high degree of selectivity in practiced hands.

Fixation

Not critical.

Solutions

0.5% Congo red in 50% alcohol

0.2% potassium hydroxide in 80% alcohol

Method

1. Take sections to water, removing pigment where necessary.
2. Stain in Congo red solution for 5 minutes.
3. Differentiate with the alcoholic potassium hydroxide solution for 3–10 seconds.
4. Wash in water, stain nuclei in alum hematoxylin, differentiate and blue.
5. Dehydrate, clear and mount.

Results

Amyloid, elastic fibers, eosinophil granules	red
Nuclei	blue

Note

Differentiation in Step 3 can be arrested in water and resumed if necessary. Over-differentiation can occur.

Congo red technique (Stokes, 1976)

In this method there is no differentiation step as Congo red is applied in an alkaline alcoholic solution. Carazzi's hematoxylin was originally used as a counterstain but any alum hematoxylin will suffice.

Fixation

Not critical.

Preparation of staining solution

Dissolve 0.5 g potassium hydroxide in 50 ml distilled water, add 200 ml absolute alcohol and add Congo red until saturated (about 3g). Stand overnight before use and discard after 3 months.

Method

1. Take sections to water, removing pigment where necessary.
2. Stain in filtered Congo red solution for 25 minutes.
3. Wash in distilled water, then running tap water for 5 minutes.
4. Counterstain nuclei in Carazzi's hematoxylin for 1 minute.
5. Blue, differentiate the hematoxylin if necessary, blue.
6. Dehydrate, clear, and mount.

Results

Amyloid, elastic tissue, eosinophil granules	red
Nuclei	blue

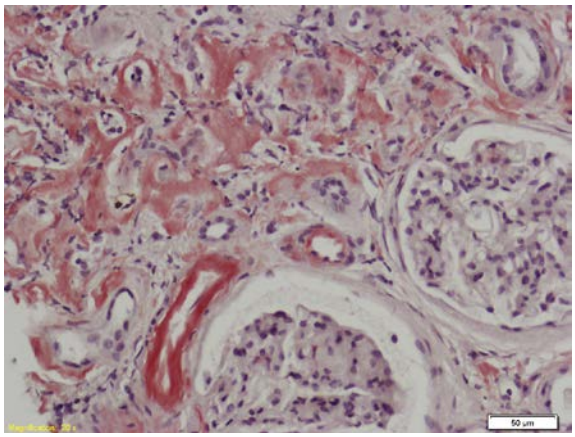

Fig. 15.4 A Congo red-stained section of kidney demonstrating amyloid deposition (red).

Sirius red

Sirius red F3B (direct red 80), is similar to Congo red and has been proposed to give a more intense staining reaction which is valuable for photographic purposes. Sirius red-stained amyloid, like Congo red, also gives green birefringence with polarized light. However, as described by Brigger & Muckle (1975), Sirius red does not display fluorescence.

Sirius red technique (Llewellyn, 1970)

Llewellyn's method is a modification of Sweat's and uses the cotton dye Sirius red F3B – not to be confused with Sirius red 4B, which does not stain amyloid. Although this method is considerably simpler than the original technique, the staining solution does not keep well and tends to precipitate out of solution.

Fixation

Not critical.

Preparation of solution

Dissolve 0.5 g Sirius red F3B in 45 ml distilled water. Add 50 ml absolute alcohol and 1 ml 1% sodium hydroxide. Stirring the solution vigorously, slowly add just sufficient 20% sodium chloride (more than 4 ml) to produce a fine precipitate when viewed against strong backlighting. Leave to stand overnight and filter.

Method

1. Take sections to water, removing pigment where necessary.

2. Stain nuclei in an alum hematoxylin, differentiate, and blue.
3. Rinse in water and then 70% ethanol.
4. Treat with Sirius red solution for 1 hour.
5. Wash in tap water for 10 minutes.
6. Dehydrate, clear and mount.

Results

Amyloid, elastic, eosinophil, and Paneth cell granules	red
Nuclei	blue

Note

The staining solution is liable to precipitation, especially if excess 20% sodium chloride is added during preparation.

Metachromatic techniques for amyloid

As we move into the next generation of technology in the modern histopathology laboratory, metachromatic techniques are now rarely used for the identification of tissue components and will only be mentioned briefly.

Methyl violet

Methyl violet was the first synthetic dye used for the demonstration of amyloid (Cornil, 1875), but the rationale of the staining reaction remains unexplained. It was thought that the mucopolysaccharide content of amyloid was responsible for the reaction, but this is now thought unlikely. The staining reaction of amyloid produces a red/purple coloration. This is also seen in other tissue components, especially mucins found in rectal biopsies, so it is not selective for amyloid. Methyl violet is a mixture of tetra-, penta-, and hexa-parasaniline, so the red/purple coloration of amyloid is probably a polychromatic reaction (Windrum & Kramer, 1957). Methyl violet-stained sections have to be mounted using an aqueous mountant since dehydration destroys the staining reaction. The amyloid from some primary amyloidosis may fail to give a positive reaction. Due to the low sensitivity and lack of specificity, this method is no longer recommended for amyloid detection and diagnosis (Westermark et al., 1999).

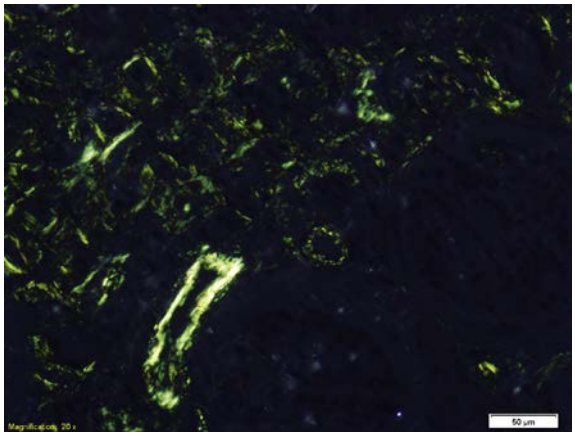

Fig. 15.5 Same field as Fig. 15.4 viewed by polarized light (crossed polarizer and analyzer). The characteristic 'green birefringence' is clearly seen against the dark background.

Crystal violet method (Hucker & Conn, 1928)

This method uses ammonium oxalate, which accentuates the polychromatic effect. Formic acid can also be used as the accentuator (Fernando, 1961).

Methyl green (Bancroft, 1963)

This method is slightly more selective; tissue sections are stained with methyl violet, and then differentiated and counterstained simultaneously with methyl green, replacing the former dye in tissue components other than amyloid.

Polarizing microscopy

As discussed earlier, when viewed using polarized light and an analyzing polarizing filter, Congo red-stained amyloid exhibits a characteristic pathognomonic bright green birefringence, usually termed 'apple-green birefringence' (Fig. 15.5). This property is shared by other β-pleated sheet proteins and appears to be specific to that conformation.

Birefringence and dichroism are optical properties of anisotropic substances, i.e. substances which have different physical properties in different directions. This property is observed in crystals, and is widely used in mineralogy as a qualitative test for the identification of various minerals. Birefringence is exhibited when an optically active substance has two differing refractive indices (RI),

so that two rays of light vibrating in perpendicular planes will travel at different velocities through the substance, producing a faster and a slower ray. The substance is said to show positive birefringence if the plane of vibration of the slow ray (greater RI) is parallel to the length of the fiber or crystal, or negative if the plane of vibration of the slow ray is perpendicular to the length of the fiber. Normally birefringent substances appear colorless (white) against a dark background when viewed through crossed polarizing filters, but where the thickness of the crystal or fiber is uniform, interference colors may be produced when the two rays are reunited in the analyzer. Such combined colors are characteristic and can be used to specifically identify the substance being viewed. For the practical details of polarizing microscopy and birefringence see Chapter 3.

Dichroism is due to light-absorbing differences along different planes of an asymmetrical substance. The spatial arrangement of light-absorbing bands is such that either light of a definite wavelength is selectively absorbed, or a change in intensity of white light occurs when light passes through the substance in certain planes. The dichroism of Congo red-stained amyloid is due to the dye molecule, whose linear molecules are bound by hydrogen bonds along the parallel folds in the β-pleated sheet protein. This produces different light-absorbing characteristics along certain planes of the fibril (Wolman & Bubis, 1965; Wolman, 1971). The different amounts of absorption of light may be seen as different colors, or different intensities of the same color (Born & Wolf, 1999).

Amyloid in unstained sections is weakly birefringent when using a strong light source; weak birefringence may also be seen following methyl violet, toluidine blue and eosin staining. Such birefringence is usually faint, unreliable and non-specific; it is of little diagnostic use. By contrast, the bright apple-green birefringence of amyloid following Congo red staining is easily visualized, highly selective and thought by many to be the most reliable diagnostic characteristic of amyloid in current use (Cohen, 1967; Pearse, 1968). This green birefringence is an intrinsic property of the amyloid fibril-Congo red complex. The thickness of the section is critical, 6–10 μm is ideal. Sections which are too thin may show faint red colors, whilst yellow birefringent colors can sometimes be seen if the section is too thick.

The use of a good microscope of high optical quality with color-corrected optics is essential to visualize faint birefringence. A revolving stage, subdued background lighting and the strongest possible light source should be used. Many laboratory microscopes do not have strong enough light sources, and sections should preferably be viewed using a modern photomicroscope equipped both with essentially perfect optics and with powerful lamps. Only by using such a setup can the smallest of amyloid deposits be appreciated.

Apple-green birefringence is also given by certain other filamentous structures, most notably the neurofibrillary tangles characteristic of Alzheimer's disease and certain other degenerative brain diseases, as well as the intracellular inclusions seen in adrenal cortical cells (Eriksson & Westermark, 1990). Whilst these structures fulfill many of the characteristics of amyloid, they are not currently considered to be amyloid, but the matter remains under debate (Westermark et al., 2005). Green birefringence is also given by cellulose and chitin, both of which avidly bind Congo red. They are easily distinguished from amyloid on morphological grounds. Occasionally other tissues may appear to give green birefringence, e.g. dense collagen. This can usually be distinguished from amyloid by its whiter color, and the difference can be emphasized by the use of sections cut at the recommended thickness.

As mentioned previously, Congo red is a fluorescent dye, and provided that sections have been mounted in a non-fluorescent mountant this property can be used to detect small amyloid deposits. The fluorescence should not be considered as specific for amyloid as is the apple-green birefringence seen with polarizing microscopy (Puchtler & Sweat, 1965; Westermark et al., 1999).

Acquired fluorescence methods

The ability of amyloid to fluoresce following treatment with fluorochromic dyes was discovered by Chiari (1947), although little use was made of this property until Vassar & Culling (1959) recommended the basic fluorochrome dye thioflavine T. This method has the advantage of not requiring microscopic differentiation and, save for staining of renal tubular myeloma casts and mast cell granules, specificity for amyloid was claimed.

Thioflavine T staining has enjoyed considerable popularity as a screening method for amyloid. The intensity of fluorescence allows good visualization of minimal deposits and has been described as an easier, more sensitive method of amyloid detection (Picken & Herrera, 2015). It is not completely specific for amyloid as many other tissue components also have an affinity for the dye (Cooper, 1969). These include fibrinoid, arteriolar hyaline, keratin, intestinal muciphages, Paneth cells and zymogen granules. The addition of 0.4 M magnesium chloride to a 0.1% thioflavine T solution at pH 5.7 is claimed to improve selectivity by competitive ionic inhibition (Mowry & Scott, 1967). Similar results are obtained by using thioflavine T at pH 1.4, favoring the reaction of the blue fluorescing dye component responsible for fluorescence of amyloid (Burns et al., 1967). The mechanism of binding thioflavine T to amyloid is not known but in vitro studies with purified amyloid fibrils and synthetic amyloids show that the dye interacts with the quaternary structure of the β-pleated sheet, rather than with protein moieties, and so the binding is not dependent on any amino acid sequence (LeVine, 1995).

A related fluorochrome, thioflavine S, has been widely used for the demonstration of amyloid (Schwartz, 1970). However, it is considered to be non-specific and is not recommended (Puchtler et al., 1985).

Thioflavine T method (Vassar & Culling, 1959)

Fixation

Not critical.

Solution

1% aqueous thioflavine T.

Method

1. Take sections to water, removing pigment where necessary.
2. Treat with alum hematoxylin solution for 2 minutes.
3. Wash in water and stain in thioflavine T solution for 3 minutes.
4. Rinse in water and differentiate excess fluorochrome from background in 1% acetic acid for 20 minutes.
5. Wash well in water, dehydrate, clear, and mount in a non-fluorescent mountant.

Results

Using a UV light source (mercury vapor lamp), UG1 Exciter filter, BG38 red suppression filter, and K430 barrier filter: amyloid, elastic tissue, etc. exhibit silver-blue fluorescence.
Using blue light fluorescence quartz-iodine or mercury vapor lamp with BG12 exciter filter and K530 barrier filter: amyloid, elastic tissue, etc. exhibit yellow fluorescence.

Notes

a. Step 2 quenches nuclear autofluorescence.
b. The mountant must be non-fluorescent, e.g. glycerine-saline (1:9 parts) or DPX. Avoid Canada balsam which autofluoresces.
c. Thioflavine T deteriorates, especially if kept in sunlight, as do the stained sections on prolonged storage.

pH 1.4 Thioflavine T (Burns et al., 1967)

Acid pH increases the selectivity by favoring the fluorochromic fraction binding to amyloid while depressing non-amyloid fluorochrome staining.

Method

As above but use a freshly prepared solution of 0.5% thioflavine T in 0.1 M hydrochloric acid.

Results

Amyloid, Paneth cells, and oxyntic cells exhibit silver-blue or yellow fluorescence according to filters used.

Miscellaneous methods

Many dyes, notably alcian blue and toluidine blue, commonly used for the identification of mucopolysaccharides have been used on amyloid-containing tissue sections to histochemically substantiate the

mucopolysaccharides frequently found in biochemical assays of amyloid tissue extracts. In most instances the results have been disappointing. Uptake of these dyes is poor and variable. Differing electrolyte concentrations and partial pepsin digestion may enhance alcian blue uptake and toluidine blue metachromasia, but staining is never strong and interpretation is difficult. The variable periodic acid-Schiff's staining of amyloid, which is often intense, was thought to indicate the presence of carbohydrate within amyloid fibrils, but this has been disproved. The glycoprotein AP component is now thought to be the origin of this positivity. Alcian blue borax, with a celestine blue hemalum and van Gieson counterstain, elegantly demonstrates some amyloid however the method is not specific (Lendrum et al., 1972).

There are several silver impregnation methods which have been used for the demonstration of amyloid in the central nervous system tissue for the detection of amyloid-containing plaques and neurofibrillary tangles in Alzheimer's disease (Lamy et al., 1989; Wisniewski et al., 1989; Wilcock et al., 1990; Vallet et al., 1992). These are discussed in Chapter 18.

Fibril extraction

Fibril extraction is a tool which utilizes small amounts of unfixed tissue to identify the amyloid fibril type and is especially useful when no other material is available. Small amounts of unfixed tissue are washed in a series of buffers, homogenized in saline, centrifuged and washed several times, leaving a suspension and a pellet of fibrils. These are then subjected to drying in layers for Congo red staining and immunohistochemistry. The optical density of the suspension is measured and SDS-PAGE, immunoblotting and SAP electro-immunoassays are carried out. Polypeptide analysis of the fibril isolates by *N*-terminal amino acid sequencing is also performed when necessary (Tennent, 1999).

Immunohistochemistry for amyloid

Immunohistochemistry (IHC) is a widely used method in histopathology for identification of tissue disease in formalin-fixed paraffin wax embedded (FFPE) samples, and can be used to determine the amyloid fibril type in most cases (Fig. 15.6). Due to the distinct protein nature of amyloid, antibodies can recognize specific epitopes on amyloid fibrils and on associated non-fibrillar constituents such as amyloid P component (AP) and proteoglycans. Some amyloid epitopes can be masked by fixation of the tissue due to the cross-linking of the amino acid side groups which mask the antigenic site. It is thought that the β-pleated conformation of the amyloid can 'hide' some antigenic sites and for identification of amyloid of transthyretin (TTR) type, oxidation and high molarity guanidine treatments (Costa et al., 1986) are needed.

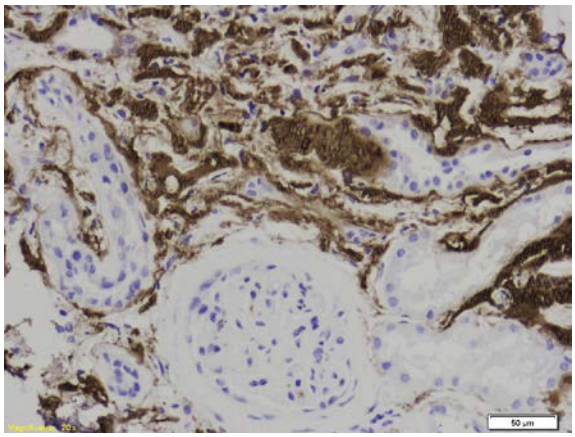

Fig. 15.6 Immunoperoxidase-DAB stained section of the same tissue as used in Fig. 15.4 using antiserum to LECT2 antibody.

AP is found in a variable proportion of all amyloid fibrils. It is a non-fibrillar component which does not have a β-pleated conformation, so antisera raised against AP can be used to corroborate the presence of amyloid. However, AP is also found naturally in basement membranes and other tissue components which will also bind antiserum raised against AP, so it is not a specific marker for amyloid. In early attempts using IHC to identify all the fibril types, various antigen retrieval methods were used with varied success. Nowadays, retrieval methods are commonplace in most laboratories and as each antibody differs in the epitope which it recognizes, it is important to try the whole range of available antigen recovery methods for each antiserum appropriate for each laboratory's

practice. However, in the author's experience, antigen retrieval is of little use for the detection of amyloid with the exception of TTR amyloid. As well as testing all the retrieval methods, the correct way to evaluate the specificity of immunoreactions is to absorb antiserum with its specific antibody (Westermark et al., 1999). Antisera to all known amyloid-forming proteins are commercially available, and most are reliable in identifying the different fibrils (Linke, 2015a, 2015b).

Differentiation between AA and AL amyloidosis is important clinically, and patients with these diseases are likely to be seen occasionally at most hospitals. Characterization of the rarer polyneuropathy-related amyloid types ATTR, AApoAI and AGel, and other hereditary types such as ALys and AFib, may reasonably be referred to more specialist centers. The context or clinical presentation of the patient and disease should be considered when trying to type the amyloid by IHC so that informed choices regarding sites of tissue biopsies and use of relevant antisera can be made.

In AL amyloidosis, about 70% of kappa and lambda types are identifiable, leaving 30% of 'probable' AL type, regardless of the methods or retrieval recoveries used. This is due to the amyloid fibrils being formed from the variable light chain fragment of the immunoglobulin molecule. Patients with AL amyloidosis may have high concentrations of free light chains in the serum; these can cause high background staining with kappa and lambda antisera, making interpretation difficult (Pepys, 1992).

It is exceptionally rare, although not completely unknown, for a patient to have two types of amyloid. When there is staining of amyloid deposits with two amyloid fibril antisera by IHC, we would suggest that proteomics should be undertaken to determine the fibril type (Mahmood et al., 2014).

The demonstration of prion-derived amyloid deposits, APrP, is difficult and most cases are referred to the Creutzfeldt-Jakob Disease Surveillance Unit which has vigorous IHC criteria (Bell et al., 1997). It is important to follow these criteria so as to avoid false-positive interference from normal prion protein, and it should be noted that the proteins within all amyloid deposits differ from the normal precursor proteins largely by alteration of conformation, rather than any antigenically determinable character.

Laser microdissection-proteomics for typing amyloid

Proteomics is a high sensitivity, mass spectrometric method for identifying proteins. Laser capture microdissection (LCMD) allows one to precisely remove an area of interest from a tissue section. This can now be coupled with a proteomics approach to offer histologists a powerful tool to identify proteins in both freshly prepared and FFPE sections. Although slides specifically made for LCMD can be obtained, it is also possible to extract material from previously cut unstained sections (archived as a matter of routine), allowing work to be carried out retrospectively to type amyloid. This approach was pioneered at the Mayo Clinic, USA (Vrana et al., 2009), and is particularly useful when IHC is challenging. A schematic diagram (Fig. 15.7) depicts the simplified laser microdissection-proteomics procedure outlined below.

A FFPE section is mounted onto a slide, dewaxed and stained with Congo red. The amyloid is visualized by its characteristic apple-green birefringence and the fluorescent properties of the Congo red dye. Using a fluorescent laser capture microscope (Fig. 15.8), the area of Congo red-positive material is excised by the laser and collected into a 0.5ml tube, where it is reduced and carbamidomethylated to protect cysteines. The tissue sample is then digested with the proteolytic enzyme trypsin. This generates a complex mixture of peptides arising from each of the proteins in the tissue. The mixture is chromatographed on a reverse phase high-performance liquid chromatography (HPLC) column to separate the tryptic peptides, which are then analyzed directly by electrospray mass spectrometry (MS). MS generates information about the molecular mass and sequence for each of the peptides in the mixture. The MS-derived data are then collated and compared against a database of all proteins using a search

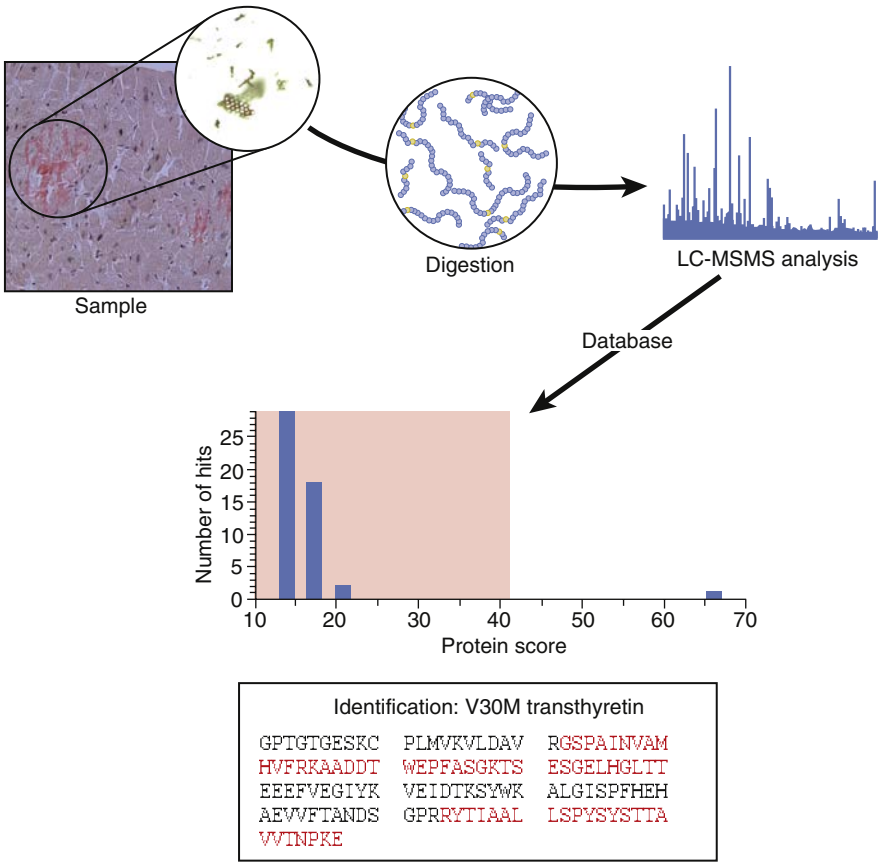

Fig. 15.7 Diagrammatic representation of the simplified LCDM method.

engine such as MASCOT, or SCAFFOLD (Fig. 15.9) which identifies the protein(s) present in tissue by using a probability-based algorithm. This LCMD-proteomics approach can not only identify the class of proteins in tissues, allowing us to type the amyloid (e.g. ATTR or AL), but also identifies novel amyloidogenic proteins (cf. LECT2) as well as protein variants found in TTR such as M30V or V122I. This approach has been useful in identifying fibrinogen α variants in only two glomeruli.

Although LCMD-proteomics requires a substantial investment both in terms of equipment and staff, it is rapidly becoming the method of choice for typing amyloid tissue and for the identification of novel amyloidogenic proteins. Within 10 years, this approach will be common in leading specialist centers for the typing of amyloid fibrils.

Evaluation of methods

The 'gold standard' for amyloid demonstration remains the use of Congo red staining with 'apple-green' birefringence when viewed with cross polarized light. The preferred method for paraffin wax-embedded material remains that of Puchtler. Thioflavine T and other fluorescent dyes may offer an alternative to Congo red, but they may be less selective, as other hyaline and fibrinoid fibers also give positive results.

Positive IHC with antisera to SAP may be useful when used in conjunction with Congo red staining, although it must be remembered that elastin and collagen fibers will also stain positively. It is of value to identify the type of amyloid present using IHC, as the success of treatment may well depend on such identification.

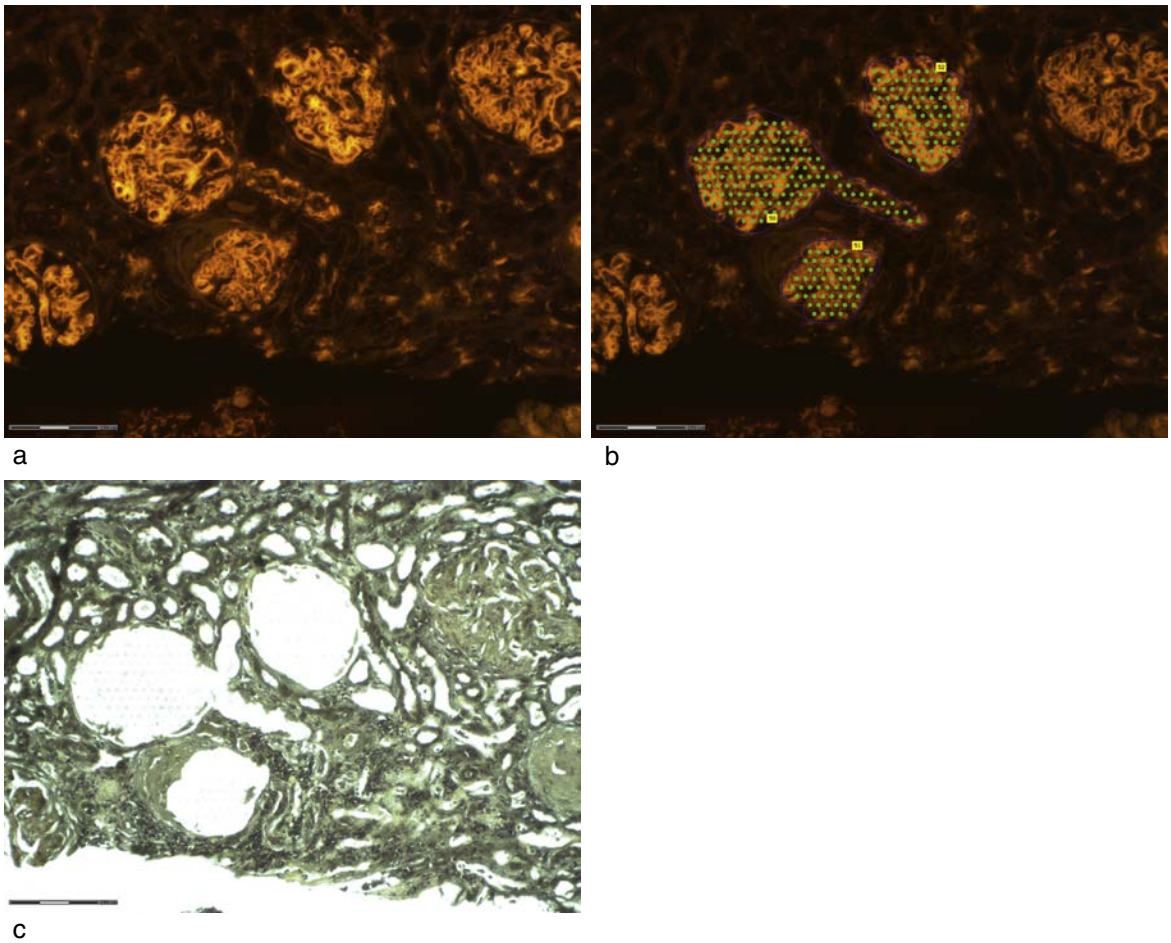

Fig. 15.8 (a) Amyloid stained by Congo red and viewed under TRITC filters. (b) Positive areas of amyloid are identified, drawn around and selected. (c) The tissue after microdissection.

The future

New therapies are constantly being investigated for the treatment of amyloidosis. At the NAC, a bis-D-proline compound, CPHPC, which depletes serum amyloid P component (SAP), the circulating precursor of AP in amyloid deposits, has been coupled with subsequent infusion of antibodies to human SAP. This therapy triggers a potent, complement-dependent, macrophage-derived giant cell reaction which swiftly removes massive murine visceral amyloid deposits without adverse effect (Bodin et al., 2010). This novel approach has recently been translated to the treatment of patients with great success in early phase clinical studies (Richards et al., 2015). The unprecedented capacity of this novel combined therapy to eliminate amyloid deposits may be applicable to all forms of systemic and localized amyloidosis in the future. Further research has found that this approach may also be applicable to Alzheimer's disease and cerebral amyloid angiopathy (Al-Shawi et al., 2016).

LCMD-proteomics is proving to be a valuable technique and is becoming more popular in leading specialist laboratories. However, it must be noted that proteomics should be considered to be complimentary to Congo red staining and IHC, and cannot yet be considered the new 'gold standard' for the typing of amyloid. Further bio-informatic

Probability Legend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

Bio View:
Identified Proteins (235)
Including 0 Decoys

#	Visible?	Starred?		Accession Number	Molecular weight	Protein Grouping Ambiguity	Patient No. 1636	Patient No. 5503
1	☑	★	Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3	APOA4_HUMAN	45 kDa		30	18
2	☑	★	Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1	APOE_HUMAN	36 kDa		23	16
3	☑	★	Serum amyloid P-component OS=Homo sapiens GN=APCS PE =1 SV=2	SAMP_HUMAN	25 kDa		10	7
4	☑	★	Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2	IGHA1_HUMAN	38 kDa		10	6
5	☑	★	Ig gamma-1 chain C region OS= Homo sapiens GN=IGHG1 PE=1 SV=1	IGHG1_HUMAN	36 kDa	★	9	7
6	☑	★	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	APOA1_HUMAN	31 kDa		9	4
7	☑	★	Immunoglobulin lambda-like polypeptide 5 OS=Homo sapiens GN=IGLL5 PE=2...	IGLL5_HUMAN	23 kDa	★	8	4
8	☑	★	Ig kappa chain C region OS=Homo sapiens GN=IGKC PE =1 SV=1	IGKC_HUMAN	12 kDa		6	5
9	☑	★	Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3	IGHM_HUMAN	49 kDa	★	6	11
10	☑	★	Ig delta chain C region OS=Homo sapiens GN=IGHD PE=1 SV=2	IGHD_HUMAN	42 kDa		5	1
11	☑	★	Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=2	IGHG3_HUMAN	41 kDa	★	4	4
12	☑	★	Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1	LYSC_HUMAN	17 kDa		4	2
13	☑	★	Insulin-like growth factor-binding protein complex acid labile subunit OS=Hom...	ALS_HUMAN (+1)	66 kDa		3	1
14	☑	★	Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1	TTHY_HUMAN	16 kDa		3	
15	☑	★	Ig lambda chain V-I region HA OS= Homo sapiens PE=1 SV=1	LV102_HUMAN	12 kDa		3	1
16	☑	★	Ig kappa chain V-III region SIE OS=Homo sapiens PE=1 SV=1	KV302_HUMAN (+3)	12 kDa		2	3
17	☑	★	Ig kappa chain V-IV region Len OS=Homo sapiens PE=1 SV=2	KV402_HUMAN	13 kDa		2	1
18	☑	★	Ig heavy chain V region 5A OS=Carassius auratus PE=4 SV=1	HV05_CARAU	13 kDa		2	1
19	☑	★	Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1	GELS_HUMAN	86 kDa		1	11
20	☑	★	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1	LAC2_HUMAN	11 kDa	★	1	3
21	☑	☆	Collagen alpha-3(VI) chain OS=Homo sapiens GN=COL6A3 PE=1 SV=5	CO6A3_HUMAN	344 kDa		84	83

Fig. 15.9 A representative analysis using SCAFFOLD software (SCAFFOLD's Samples View, printed with permission from Proteome Software, Inc. Copyright 2017.)

development and standardization is required before it can be widely and reliably applied in the routine laboratory (Gilbertson et al., 2015b).

DNA extraction and gene sequencing can be carried out on FFPE samples, and with the arrival of next generation sequencing (NGS) in leading laboratories this technique may be a method of choice for diagnosing hereditary amyloidosis. However, this technique would not be useful for diagnosing AA and AL amyloidosis.

A new approach in identifying all amyloid proteins may be the use of LCDM and proteomics on decellularised tissue (Mazza et al., 2016). After decellularisation the amyloid framework remains, (Mangione, 2017), giving only the amyloidogenic protein when LCDM and proteomics is performed. This technique is still in the experimental stage but may be a technique of the future.

Acknowledgments

Special thanks to friends, colleagues and patients at the National Amyloidosis Centre for their support and encouragement.

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16 Traditional stains and modern techniques for demonstrating microorganisms in histology

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Introduction

Microorganisms (or microbes) are organisms which can only be individually seen by microscopy. Many do not cause disease in humans and act as normal colonizers of human hosts. Complex interactions between pathogens, which are capable of causing diseases, the host and the environment lead to clinical infections. Pathogens fall into five main groups:

- Viruses
- Bacteria
- Fungi
- Protozoa
- Helminths.

With the advent of new and broader spectrum antibiotics, improved environmental hygiene, and advances in microbiological techniques it was widely expected that the need for diagnosis of infectious agents in tissue would diminish in importance. This assumption underestimated the infinite capacity of infectious agents for genomic variation, enabling them to develop antimicrobial resistance and exploit new opportunities to spread infections which are created when host defenses become compromised. The following are currently the most important factors influencing the presentation of infectious diseases:

- The increased mobility of the world's population through tourism, immigration and international commerce has distorted natural geographic boundaries to infection, exposing weaknesses in host defenses, and in knowledge.
- Immunodeficiency states occurring either as part of an infection, e.g. Human Immunodeficiency Virus (HIV) which causes acquired immune deficiency syndrome (AIDS), or as an iatrogenic disease. As treatment becomes more aggressive, depression of the host's immunity occurs, enabling organisms of low virulence to become life-threatening, and may allow latent infections, accrued throughout life, to reactivate and spread.
- Emerging, re-emerging and antibiotic-resistant organisms such as the tubercle bacillus and staphylococcus are a constant and growing threat.
- Adaptive mutation occurring in microorganisms allows them to jump species barriers and exploit new physical environments. Such adaptation allows infections to evade host defenses and resist agents of treatment.
- Bioterrorism has become an increasing concern. The world's public health systems and primary healthcare providers must be prepared to address varied biological agents, including pathogens which are rarely seen in developed countries. High-priority agents include organisms which pose a risk to national security because they:
  - Can easily be disseminated or transmitted from person to person.
  - Cause high mortality, with potential for a major public health impact.
  - May cause public panic and social disruption, and require special action for public health preparedness.

  The following are listed by the Centers for Disease Control and Prevention (CDC) in the United States as high-risk biological agents:
  - Anthrax
  - Smallpox
  - Botulism
  - Tularemia
  - Viral hemorrhagic fever (various).

These factors, acting singly or together, provide an ever-changing picture of infectious disease where clinical presentation may involve multiple pathological processes, unfamiliar organisms, and modification of the host response by a diminished immune status.

Size

The term 'microorganism' has been interpreted liberally in this chapter. Space limitation precludes a comprehensive approach to the subject, and the reader is referred to additional texts e.g. von Lichtenberg (1991) and Mandell et al. (2014) for greater depth. The organisms in Table 16.1 are discussed and techniques for their demonstration are described.

Safety

Most infectious agents are rendered harmless by direct exposure to formal saline. Standard fixation procedures should be sufficient to kill microorganisms, one exception being material from those with Creutzfeldt-Jakob disease (CJD) and other prion diseases. It has been shown that well-fixed tissue, paraffin-processed blocks and stained slides from CJD cases remain infectious when introduced into susceptible animals. Treatment of fixed tissue or slides in 96% formic acid for 1 hour followed by copious washing inactivates this infectious agent without adversely affecting section quality (Brown et al., 1990). Laboratory safety protocols should cover infection containment in all laboratory areas and the mortuary, or necropsy area, where handling unfixed material is unavoidable. When available, unfixed tissue samples should be sent for microbiological culture, as this offers the best chance of rapid and specific identification of etiological agents, even when heavy bacterial contamination may have occurred.

Table 16.1 Size of organisms

Organisms	Size
Viruses	20–300 nm
Mycoplasmas	125–350 nm
Chlamydia	200–1000 nm
Rickettsia	300–1200 nm
Bacteria	1–14 μm
Fungi	2–200 μm
Protozoa	1–50 μm
Metazoans	3–10 mm

General principles of detection and identification

The diagnosis of illness from infectious diseases generally starts with clinical presentation of the patient, and in most cases a diagnosis is made without a tissue sample being taken. Cases which rely on tissue diagnosis range from autopsy specimens, where material maybe plentiful and sampling error presents little problem, through to cytology samples where cellular material is often scarce and lesions may be easily missed. A full clinical history is always important, especially details of the patient's ethnic origin, immune status, any recent history of foreign travel and current medication.

The macroscopic appearance of tissue may often give a clear diagnosis of infection. Those with frank pus, abscess formation, cavitation, hyperkeratosis, demyelination, pseudo-membrane formation, focal necrosis and granulomas can provide evidence of infection. These appearances are often non-specific but occasionally in hydatid cyst disease or some helminth infestations the appearances are diagnostic.

The microscopic appearance of routine stained sections at low-power magnification often reveals indirect evidence of the presence of infection, e.g. neutrophil or lymphocytic infiltrates, granuloma formation, micro-abscesses, eosinophilic aggregates, Charcot-Leyden crystals and caseous necrosis. Some of these appearances may be sufficiently reliable to provide an initial, or at least provisional diagnosis, and allow treatment to be started, even if the precise nature of the suspect organism is never identified, particularly in the case of tuberculosis.

At the cellular level, the presence of giant cells e.g. Warthin-Finkeldy or Langhans' type may indicate measles or tuberculosis respectively. Other cellular changes which include intra-cytoplasmic edema of koilocytes, acantholysis, spongiform degeneration of the brain, margination of chromatin, syncytial nuclear appearance, 'ground-glass' changes in the nucleus or cytoplasm, or inclusion bodies, can indicate infectious etiology. At some stage in these processes, suspect organisms may be visualized.

It should be understood, a well-performed hematoxylin and eosin (H&E) method will stain many organisms. Papanicolaou stain and Romanowsky stains, e.g. Giemsa, will also stain organisms together with their cellular environment. Other infectious agents are poorly visualized by routine stains and require special techniques to demonstrate their presence. This may be due to the small size of the organism, as in the case of viruses, where electron microscopy is needed. Alternatively, the organism may be hydrophobic or weakly charged, as with mycobacteria, spirochetes and cryptococci, in which case the use of specific histochemical methods is required for their detection. When organisms are few in number, fluorochromes may be used to increase the microscopic sensitivity of a technique. Finally, the following two techniques offer the possibility of specific identification of microorganisms which extend to the appropriate strain level.

Immunohistochemistry (IHC)

Immunohistochemistry (see Chapter 19) is now a routine and invaluable procedure in the histopathology laboratory for the detection of many microorganisms. There are many commercially available antibodies for viral, bacterial and parasitic organisms. Most methods today utilize (strept) avidin-biotin technologies. These are based on the high affinity that (strept)avidin (*Streptomyces avidinii)* and avidin (chicken egg) have for biotin. Both possess four binding sites for biotin, but due to the molecular orientation of the binding sites fewer than four molecules of biotin will actually bind. The basic sequence of reagent application consists of primary antibody, biotinylated secondary antibody, followed by either the preformed (strept) avidin-biotin enzyme complex or the avidin-biotin complex (ABC) technique or by the enzyme-labeled streptavidin. Both conclude with the substrate solution. Horseradish peroxidase and alkaline phosphatases are the most commonly used enzyme labels.

Molecular methods

The application of molecular techniques for the detection of microorganisms has arguably revolutionized the diagnosis of infection. These methods represent a rapidly expanding and exciting field, particularly when considering novel and emerging infections. However, testing must be undertaken rationally and appropriately in order to produce meaningful results (Procop, 2007). Conventional staining may lack sensitivity and specificity to detect and speciate microorganisms. Microbial culture is not viable from formalin-fixed, paraffin-embedded (FFPE) specimens. In comparison, molecular identification of pathogens is rapid with high sensitivity and specificity and can be applied to a variety of histological specimens (Rogers et al., 2009).

Common molecular techniques used include direct hybridization and nucleic acid amplification (often referred to under the umbrella term of polymerase chain reaction – PCR) (Procop, 2007). In situ hybridization (ISH) uses reporter synthetic DNA probes which hybridize and label specific RNA sequences in target microbes present in the sample. This technique is most useful when type or genus of the microorganism has been elucidated, e.g. to identify the exact species of staphylococcus or yeast. It has been used successfully to detect and accurately differentiate a range of morphologically related organisms such as *Legionella* spp., filamentous bacteria and fungi in tissue samples (Hayden et al., 2001, 2003; Isotalo et al., 2009).

PCR relies on the detection of unique regions of microbial DNA or RNA following the extraction and amplification of genetic material from specimens, and can be used to diagnose microbial infections

from autopsy tissues and surgical specimens. Whilst fresh/frozen tissues provide the best-quality nucleic acids for analysis, DNA and RNA extracted from FFPE samples can be used successfully for PCR testing. A number of specific PCRs have been developed and applied to detect a range of viruses, bacteria, fungi and parasites in histopathological specimens (Denison et al., 2011; Surat et al., 2014; Rickerts, 2016; Gebhardt et al., 2015).

Since formalin cross-links proteins and nucleic acids resulting in significant degradation, it is essential to begin processing of specimens as quickly as possible, ensuring that a 10% concentration of formalin is used for fixation, and making certain that fixation times are kept to less than 48 hours (von Ahlfen et al., 2007; Chung et al., 2008; Srinivasan et al., 2002.). Individual PCRs are useful when a particular infecting organism is suspected; in contrast, micro-array and multiplex PCR has the ability to identify a variety of related and unrelated micro-organisms simultaneously from a single sample (Fukumoto et al., 2015). Broader still, although less sensitive, are pan-bacterial and pan-fungal 16S and 18S RNA PCR probes which will detect the presence of any bacterial or fungal RNA. Further analysis and sequencing of any relevant genetic material identified is used to characterize the species.

A benefit of investigating samples using PCR analysis is the generation of quantitative data which indicate the microbial burden. This aids interpretation of results, as the presence of an organism does not necessarily mean infection. Indeed, PCR positivity may be misleading if the patient has been exposed to prior antimicrobial agents or where the microorganism persists despite clinical resolution, as is the case with many respiratory viral infections (Lehners et al., 2016). Although relatively expensive, molecular methods of diagnosis are becoming increasingly routine and available with less restrictive costs.

These techniques have a unique role to play in the identification of novel infectious diseases from histological samples, particularly at autopsy, for example, pandemic influenza virus (Shieh et al., 2010), and will continue to play a key role in the detection of emerging infections and bioterrorist attacks (Hajjeh et al., 2002) . In addition, as technology advances, it is now possible to obtain detailed genetic sequencing information which provides important information relating to microbial transmission, virulence and resistance mechanisms. This is increasingly important in an age of global communities and the advent of unprecedented antimicrobial resistance. However, further studies are still required to answer a more fundamental question, which is whether molecular testing improves patient outcomes, and this is an area for future work. In summary, molecular methods offer the ability to make a rapid and accurate diagnosis of infection of a broad range of potential pathogens. It is vital that these tests are used judiciously and interpreted with care.

Whilst modern advances in technique are important, emphasis is also placed upon the ability of the microscopist to interpret suspicious signs from a good H&E stained section. The growing number of patients whose immune status is compromised, or those who can mount only a minimal or inappropriate response to infection, further complicates the picture. This justifies speculative use of special stains, such as those for mycobacteria and fungi on tissue from such patients. It should be remembered, that for a variety of reasons, negative results for the identification of an infectious agent do not exclude its presence. In particular, administration of antibiotics to the patient before a biopsy is often the reason for failure to detect a microorganism in tissue.

Detection and identification of bacteria

When bacteria are present in large numbers, in an abscess or vegetation on a heart valve, they appear as blue-gray granular masses with an H&E stain. However, organisms are often poorly visible, and can be obscured by cellular debris. The reaction of pyogenic bacteria to the Gram stain, together with their morphological appearance (i.e. cocci or bacilli) provides the basis for a simple historical classification (Table 16.2).

Table 16.2 A simplified classification of important bacteria

Gram-positive bacteria		Gram-negative bacteria		
Cocci	**Bacilli**	**Cocci**	**Bacilli**	**Coccobacilli**
Staphylococcus	Bacillus	Neisseria	Escherichia	Brucella
	Clostridium		Klebsiella	Bordetella
Streptococcus	Corynebacterium		Salmonella	Haemophilus
(inc. Pneumococcus)	Mycobacteria (weak+)		Shigella	
	Lactobacillus (commensal)		Proteus	
	Listeria		Pseudomonas	
			Vibrio	
			Pasteurella	

Use of control sections

The use of known positive control sections with all special stain methods for demonstrating microorganisms is essential. Results are unsafe in the absence of positive controls, and should not be considered valid. The control section should be appropriate, where possible, for the suspected organism. For example, a pneumocystis-containing control should be used for demonstrating *Pneumocystis jiroveci* (previously called *carinii*). A Gram control should contain both Gram-positive and Gram-negative organisms. Post-mortem tissues have previously been a good source of control material, although medico-legal issues have now limited this in some countries. Alternatively, a suspension of Gram-positive and Gram-negative organisms can be injected into the thigh muscle of a rat shortly before it is sacrificed for some other purpose. Gram-positive and Gram-negative organisms can also be harvested from microbiological plates, suspended in 10% neutral buffered formalin (NBF), centrifuged, and small amounts mixed with minced normal kidney, then chemically processed along with other tissue blocks (Swisher & Nicholson, 1989).

The Gram stain

In spite of more than a century since Gram described his technique in 1884, its chemical rationale remains obscure. Staining is due to a mixture of factors, the most important being cell wall thickness, chemical composition and the functional integrity of the cell walls of Gram-positive bacteria. When these bacteria die, they become Gram negative. The following procedure is only suitable for the demonstration of bacteria in smears of pus and sputum. It may be of value to the pathologist in the necropsy room where a quick technique such as this may enable rapid identification of the organism causing a lung abscess, wound infection, septicemic abscess or meningitis.

Gram method for bacteria in smears (Gram, 1884)

Method

1. Fix dry film by passing it three times through a flame or placing on a heat block.
2. Stain for 15 seconds in 1% crystal violet or methyl violet, and then pour off excess.
3. Flood for 30 seconds with Lugol's iodine, pour off excess.
4. Flood with acetone for no more than 2–5 seconds, wash with water immediately.
5. Alternatively decolorize with alcohol until no more stain comes out. Wash with water.
6. Counterstain for 20 seconds with dilute carbol fuchsin, or freshly filtered neutral red for 1–2 minutes.
7. Wash with water and carefully blot section until it is dry.

Results

Gram-positive organisms	blue/black
Gram-negative organisms	red

Modified Brown-Brenn method for Gram-positive and Gram-negative bacteria in paraffin sections (Churukian & Schenk, 1982)

Sections

Formalin-fixed, 4–5 µm, paraffin wax embedded sections.

Solutions

Crystal violet solution (commercially available)

Crystal violet, 10% alcoholic	2 ml
Distilled water	18 ml
Ammonium oxalate, 1%	80 ml

Mix and store; always filter before use.

Modified Gram's iodine commercially available, or

Iodine	2 g
Potassium iodide	4 g
Distilled water	400 ml

Dissolve potassium iodide in a small amount of the distilled water, add iodine and dissolve; add remainder of distilled water.

Ethyl alcohol-acetone solution

Ethyl alcohol, absolute	50 ml
Acetone	50 ml

0.5% basic fuchsin solution (stock) commercially available, or

Basic fuchsin or pararosaniline	0.5 g
Distilled water	100 ml

Dissolve with aid of heat and a magnetic stirrer.

Basic fuchsin solution (working)

Basic fuchsin solution (stock)	10 ml
Distilled water	40 ml

Picric acid-acetone

Picric acid	0.1 g
Acetone	100 ml

Note

With concerns over the explosiveness of dry picric acid in the lab, it is recommended that you purchase the picric acid-acetone solution pre-made. It is available through most histology suppliers.

Acetone-xylene solution

Acetone	50 ml
Xylene	50 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain with filtered crystal violet solution for 1 minute.
3. Rinse well in distilled water.
4. Place in iodine solution for 1 minute.
5. Rinse in distilled water, blot slide but NOT the tissue section.
6. Decolorize by dipping in alcohol-acetone solution until the blue color stops running. (One to two dips only!)
7. Counterstain in working basic fuchsin for 1 minute. Be sure to agitate the slides well in the basic fuchsin before starting the timer.
8. Rinse in distilled water and blot slide but not section.
9. Dip in acetone, one dip.
10. Dip in picric acid-acetone until the sections have a yellowish-pink color.
11. Dip several times in acetone-xylene solution. At this point, check the control for proper differentiation. (Go back to picric acid-acetone if you need more differentiation.)
12. Clear in xylene and mount.

Results

Gram-positive organisms, fibrin, some fungi, Paneth cell granules, kerato-hyalin, and keratin	blue
Gram-negative organisms	red
Nuclei	red
Other tissue elements	yellow

Note

Do not allow the tissue sections to dry at any point in the staining process. If this occurs after treatment with iodine, decolorization will be difficult and uneven.

Gram-Twort stain (Twort, 1924; Ollett, 1947)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

Crystal violet solution (see previous method)
Gram's iodine (see previous method)
Twort's stain

1% neutral red in ethanol	9 ml
0.2% fast green in ethanol	1 ml
Distilled water	30 ml

Mix immediately before use.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in crystal violet solution for 3 minutes.
3. Rinse in gently running tap water.

4. Treat with Gram's iodine for 3 minutes.
5. Rinse in tap water, blot dry, and complete drying in a warm place.
6. Differentiate in preheated acetic alcohol until no more color washes out (2% acetic acid in absolute alcohol, preheated to 56°C). This may take 15–20 minutes; the section should be light brown or straw colored.
7. Rinse briefly in distilled water.
8. Stain in Twort's for 5 minutes.
9. Wash in distilled water.
10. Rinse in acetic alcohol until no more red runs out of the section; this only takes a few seconds.
11. Rinse in fresh absolute alcohol, clear, and mount.

Results

Gram-positive organisms	blue/black
Gram-negative organisms	pink/red
Nuclei	red
Red blood cells and most cytoplasmic structures	green
Elastic fibers	black

Techniques for mycobacteria

These organisms are difficult to demonstrate by the Gram technique as they possess a capsule containing a long-chain fatty acid (mycolic acid) which makes them hydrophobic. The fatty capsule influences the penetration and resistance to removal of the stain by acid and alcohol (acid- and alcohol-fastness), and is variably robust between the various species which make up this group. Phenolic acid, and frequently heat, are used to reduce surface tension and increase porosity, thus forcing dyes to penetrate this capsule. The speed with which the primary dye is removed by differentiation with acid alcohol is proportional to the extent of the fatty coat. The avoidance of defatting agents or solvents, such as alcohol and xylene in methods for *Mycobacterium leprae*, is an attempt to conserve this fragile fatty capsule.

Mycobacteria are PAS positive due to the carbohydrate content of their cell walls. However, this positivity is evident only when large concentrations of the microorganisms are present. When these organisms die, they lose their fatty capsule and consequently their carbol fuchsin positivity. The carbohydrate can still be demonstrated by Grocott's methenamine silver reaction, which may prove useful when acid-fast procedures fail, particularly if the patient is already receiving therapy for tuberculosis.

A possible source of acid-fast contamination may be found growing in viscous material sometimes lining water taps and any rubber tubing connected to them. These organisms are acid- and alcohol-fast but are usually easily identified as contaminants by their appearance as clumps, or floaters, above the microscopic focal plane of the section.

Ziehl-Neelsen (ZN) stain for Mycobacterium bacilli (Kinyoun, 1915)

Sections

Formalin or fixative other than Carnoy's, paraffin wax embedded.

Solutions

Carbol fuchsin commercially available, or

Basic fuchsin	0.5 g
Absolute alcohol	5 ml
5% aqueous phenol	100 ml

Mix well and filter before use.

Acid alcohol

Hydrochloric acid	10 ml
70% alcohol	1000 ml

Methylene blue solution (stock) commercially available, or

Methylene blue	1.4 g
95% alcohol	100 ml

Methylene blue solution (working)

Methylene blue (stock)	10 ml
Tap water	90 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in carbol fuchsin solution for 30 minutes.
3. Wash well in tap water.
4. Differentiate in acid alcohol until solutions are pale pink. (This usually only takes 2–5 dips.)
5. Wash in tap water for 8 minutes, then dip in distilled water.

6. Counterstain in working methylene blue solution until sections are pale blue.
7. Rinse in tap water, then dip in distilled water.
8. Dehydrate, clear, and mount.

Results

Mycobacteria, hair shafts, Russell bodies, Splendore-Hoeppli immunoglobulins around actinomyces and some fungal organisms	red
Background	pale blue

Notes

a. The blue counterstain may be patchy if extensive caseation is present. Care should be taken to avoid over-counterstaining as scant organisms can easily be obscured.
b. Decalcification using strong acids can destroy acid-fastness; formic acid is recommended.
c. Victoria blue can be substituted for carbol fuchsin and picric acid for the counterstain if color blindness causes a recognition problem.

Fluorescent method for Mycobacterium bacilli (Kuper & May, 1960)

Sections

Formalin fixed, paraffin wax embedded.

Solution

Auramine O	1.5 g
Rhodamine B	0.75 g
Glycerol	75 ml
Phenol crystals (liquefied at 50°C)	10 ml
Distilled water	50 ml

Method

1. Deparaffinize (1-part groundnut oil and 2 parts xylene for *M. leprae*).
2. Pour on preheated (60°C), filtered staining solution for 10 minutes.
3. Wash in tap water.
4. Differentiate in 0.5% hydrochloric acid in alcohol for *M. tuberculosis*, or 0.5% aqueous hydrochloric acid for *M. leprae*.
5. Wash in tap water, 2 minutes.
6. Eliminate background fluorescence in 0.5% potassium permanganate for 2 minutes.
7. Wash in tap water and blot dry.
8. Dehydrate (not for *M. leprae*), clear and mount in a fluorescence-free mountant.

Results

Mycobacteria	golden yellow (using blue light fluorescence below 530 nm)
Background	dark green

Note

The advantage of increased sensitivity of this technique is offset by the inconvenience of setting up the fluorescence microscope. Preparations fade over time, as a result of their exposure to UV light.

Modified Fite method for *Mycobacterium leprae* and Nocardia

Fixation

10% neutral buffered formalin (NBF).

Sections

Paraffin wax sections at 4–5 µm.

Solutions

Carbol fuchsin solution commercially available, or
0.5 g basic fuchsin dissolved in 5 ml of absolute alcohol; add 100 ml of 5% aqueous phenol. Mix well and filter before use. Filter before each use with #1 filter paper.

5% sulfuric acid in 25% alcohol

25% ethanol	95 ml
Sulfuric acid, concentrated	5 ml

Methylene blue stock solution, commercially available, or

Methylene blue	1.4 g
95% alcohol	100 ml

Methylene blue working solution

Stock methylene blue	5 ml
Tap water	45 ml
Xylene-peanut oil	1 part oil: 2 parts xylene

Method

1. Deparaffinize in two changes of xylene-peanut oil for 6 minutes each.
2. Drain slides vertically on paper towel and wash in warm, running tap water for 3 minutes.

(The residual oil preserves the sections and helps accentuate the acid fastness of the bacilli.)
3. Stain in carbol fuchsin at room temperature for 25 minutes. (Solution may be poured back into bottle and reused.)
4. Wash in warm, running tap water for 3 minutes.
5. Drain excess water from slides vertically on paper towel.
6. Decolorize with 5% sulfuric acid in 25% alcohol, two changes of 90 seconds each. (Sections should be pale pink.)
7. Wash in warm, running tap water for 5 minutes.
8. Counterstain in working methylene blue, one quick dip. (Sections should be pale blue.)
9. Wash in warm, running tap water for 5 minutes.
10. Blot sections and dry in 50–55°C oven for 5 minutes.
11. Once dry, one quick dip in xylene.
12. Mount with permanent mountant.

Results (Fig. 16.1)

Acid-fast bacilli including *M. leprae*	bright red
Nuclei and other tissue elements	pale blue

Quality control/note

Do not overstain with methylene blue and do not allow sections to dry between carbol fuchsin and acid alcohol.

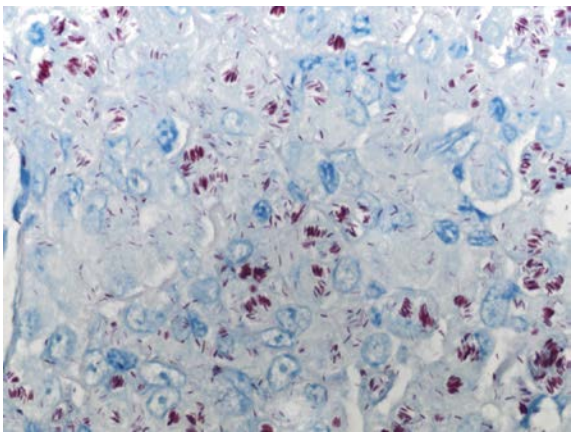


Fig. 16.1 The modified Fite procedure is necessary to demonstrate *Mycobacterium leprae* due to the organism's fragile, fatty capsule.

Techniques for other important bacteria

Cresyl violet acetate method for Helicobacter species

Sections

Formalin fixed, paraffin wax embedded.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Filter 0.1% cresyl violet acetate onto slide or into Coplin jar for 5 minutes.
3. Rinse in distilled water.
4. Blot, dehydrate rapidly in alcohol, clear, and mount.

Results

Helicobacter and nuclei	blue/violet
Background	shades of blue/violet

Notes

This simple method allows good differentiation between Helicobacter sp. and other organisms.

Gimenez method for *Helicobacter pylori* (Gimenez, 1964; McMullen et al., 1987)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

Buffer solution (phosphate buffer at pH 7.5, or 0.1 M)

0.1 M sodium dihydrogen orthophosphate	3.5 ml
0.1 M disodium hydrogen orthophosphate	15.5 ml

Stock carbol fuchsin

Commercial cold acid-fast bacilli stain, or basic fuchsin	1 g
Absolute alcohol	10 ml
5% aqueous phenol	10 ml

Filter before use.

Working carbol fuchsin

Phosphate buffer	10 ml
Stock carbol fuchsin	4 ml

Filter before use.

Malachite green

Malachite green	0.8 g
Distilled water	100 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in working carbol fuchsin solution for 2 minutes.
3. Wash well in tap water.
4. Stain in malachite green for 15–20 seconds.
5. Wash thoroughly in distilled water.
6. Repeat steps 4 and 5 until section is blue-green to the naked eye.
7. Blot sections dry, and complete drying in air.
8. Clear and mount.

Results

Helicobacter pylori	red/magenta
Background	blue/green

Note

The greatest problem with this method is overstaining or irregularity of staining with Malachite green. It is also valuable in demonstrating the Legionella bacillus in post-mortem lung smears.

Toluidine blue in Sorenson's buffer for Helicobacter

Sections

Formalin fixed, paraffin wax embedded.

Solutions

Toluidine blue in pH 6.8 phosphate buffer

Sorenson's phosphate buffer, pH 6.8	50 ml
1% aqueous toluidine blue	1 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in buffered toluidine blue for 20 minutes.
3. Wash well in distilled water.
4. Dehydrate, clear, and mount.

Results

Helicobacter	dark blue against a variably blue background

Immunohistochemistry techniques for demonstrating Helicobacter species also exist.

Warthin-Starry method for spirochetes (Warthin & Starry, 1920)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

Acetate buffer, pH 3.6

Sodium acetate	4.1 g
Acetic acid	6.25 ml
Distilled water	500 ml

Silver solution

1% silver nitrate in pH 3.6 acetate buffer

Developer

Dissolve 0.3 g of hydroquinone in 10 ml pH 3.6 acetate buffer, and mix 1 ml of this solution and 15 ml of warmed 5% Scotch glue or gelatin; keep at 40°C. Take 3 ml of 2% silver nitrate in pH 3.6 buffer solution and keep at 55°C. Mix these two solutions immediately before use.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Celloidinize in 0.5% celloidin, drain and harden in distilled water for 1 minute.
3. Impregnate in preheated 55–60°C silver solution for 90–105 minutes.
4. Prepare and preheat developer in a water bath.
5. Treat with developer for $3^1/_2$ minutes at 55°C. Sections should be golden-brown at this point.
6. Remove from developer and rinse in tap water for several minutes at 55–60°C, then in buffer at room temperature.
7. Tone in 0.2% gold chloride.
8. Dehydrate, clear, and mount.

Results

Spirochetes	black
Background	golden/yellow

Note

It is wise to take a few slides through at various incubation times to ensure optimum impregnation.

Modified Steiner method for filamentous and non-filamentous bacteria (Steiner & Steiner, 1944; modified Swisher, 1987)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

1.0% uranyl nitrate commercially available, or

Uranyl nitrate	1 g
Distilled water	100 ml

1% silver nitrate

Silver nitrate	1 g
Distilled water	100 ml

Make fresh each time and filter with #1 or #2 filter paper before use.

0.04% silver nitrate

Silver nitrate	0.04 g
Distilled water	100 ml

Refrigerate and discard after 1 month.

2.5% gum mastic commercially available, or

Gum mastic	2.5 g
Absolute alcohol	100 ml

Allow to dissolve for 24 hours, then filter until clear yellow before use. Refrigerate any unused portion.

Hydroquinone

Hydroquinone	1 g
Distilled water	25 ml

Make fresh solution for each use.

Reducing solution

Mix 10 ml of 2.5% gum mastic, 25 ml of 2.0% hydroquinone and 5 ml absolute alcohol. Make just prior to use and filter with #4 filter paper; add 2.5 ml of 0.04% silver nitrate. Do not filter this solution. When the gum mastic is added, the solution will take on a milky appearance.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Sensitize sections in 1% aqueous uranyl nitrate at room temperature and place in microwave oven until solution is just at boiling point, approx. 20–30 seconds; do not boil. *Alternatively*, place in preheated 1% uranyl nitrate at 60°C in a water bath for 15 minutes, or in microwave oven and bring to almost boiling point – do not boil; 2% zinc sulfate in 3.7% formalin may be substituted.
3. Rinse in distilled water at room temperature until uranyl nitrate residue is eliminated.
4. Place in 1% silver nitrate at room temperature and microwave *until* boiling point is just reached. Do not boil. Remove from oven, loosely cover jar, and allow to stand in hot silver nitrate, 6–7 minutes; *alternatively*, preheat silver nitrate for 20–30 minutes in a 60°C water bath, add slides, and allow to impregnate for 90 minutes.
5. Rinse in three changes of distilled water.
6. Dehydrate in two changes, each of 95% alcohol and absolute alcohol.
7. Treat with 2.5% gum mastic for 5 minutes.
8. Allow to air dry for 5 minutes.
9. Rinse in two changes of distilled water. Slides may stand here while reducing solution is being prepared.
10. Reduce in preheated reducing solution at 45°C in a water bath for 10–25 minutes, or until sections have developed satisfactorily with black microorganisms against a light yellow background. Avoid intensely stained background.
11. Rinse in distilled water to stop reaction.
12. Dehydrate, clear, and mount.

Results (Fig. 16.2)

Spirochetes, cat-scratch organisms, Donovan bodies, non-filamentous bacteria of *Legionella pneumophila*	dark brown/black
Background	bright yellow to golden yellow

Notes

Bring all solutions to room temperature before using. All glassware making contact with silver nitrate should be chemically cleaned. Avoid the use of metal forceps in silver solutions. When doing a bacterial screen, Gram controls should be run along with diagnostic slides. As spirochetes take longer to develop, Gram controls should be used in addition to spirochete controls. When Gram controls have a yellow appearance, remove them to distilled water, and check under microscope for microorganisms. Return to silver solution if they are not ready, and repeat, realizing that spirochetes will take longer. Most solutions can be made in large quantities and kept in the refrigerator.

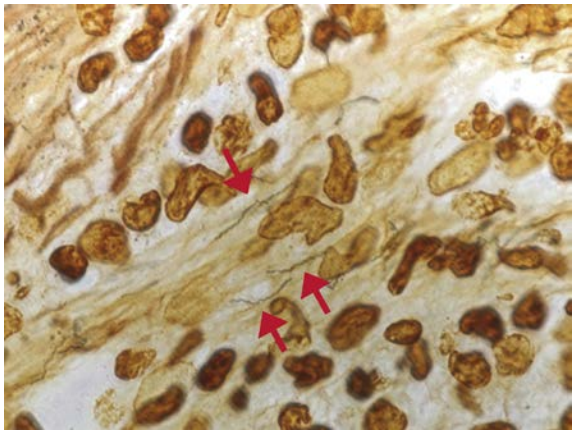


Fig. 16.2 Syphilis *Treponema pallidum* bacilli (arrowed), seen with the modified Steiner technique. The resistance to coloration is shared by Helicobacter, spirochetes and Legionella.

Some important bacteria

Staphylococcus aureus is an important and common bacterial pathogen which may be resistant to common antibiotic treatments. It usually causes skin and soft tissue infections such as boils, wound and burn infections, and can also lead to a cavitating pneumonia in children and adults. Septicemic states may occur and are associated with the formation of multiple deep foci of infection including endocarditis. Microscopically staphylococci appear as Gram-positive clusters.

Neisseria meningitidis (meningococcus) is a common cause of meningitis, and may produce a fulminating septicemia (meningococcal sepsis). Organisms can be seen in histological sections of meningococcal meningitis, but are difficult to identify because they are usually within neutrophil cytoplasm.

Neisseria gonorrhoeae (gonococcus) is the cause of gonorrhea. Organisms may be seen within polymorphs in sections of cervix, endometrium or Fallopian tubes in cases of gonorrhea but, again, are difficult to find. Members of the Neisseria family are generally difficult to see in histological sections, although easily detectable in smears of fresh pus or cerebrospinal fluid (CSF), characteristically in pairs. They are easier to detect using the Gram-Twort method.

Lactobacillus acidophilus (Döderlein's bacillus) is a normal inhabitant of the human vagina and is seen in cervical smears taken in the secretory phase of the cycle.

Corynebacterium vaginale is a short Gram-negative bacillus which may cause cervicitis, and is present in about 6% of women of childbearing age. It may be seen in cervical smears where it accumulates as blue-stained masses on the surface of squamous cells stained by Papanicolaou's method, with these cells being known as 'clue cells'.

Helicobacter pylori is frequently seen in gastric biopsies. This spiral Vibrio organism is implicated as the organism causing many cases of chronic gastritis. They are seen as small, weakly hematoxyphilic organisms (usually in clumps) in the lumina of gastric glands, often adherent to the luminal surface of the epithelial cells. With practice, these can be identified from an H&E stain. However, Warthin-Starry, Steiner, Gimenez, toluidine blue or cresyl violet acetate methods demonstrate them more clearly. A commercial specific antiserum has recently become available for their demonstration.

Clostridium difficile causes pseudomembranous colitis, an inflammation of the large bowel. This arises following the administration of broad-spectrum antibiotics; the balance of the normal anaerobic gut microflora is disturbed, allowing the organism to proliferate unchecked. *C. difficile* is difficult to stain but the 'volcano lesions' of purulent necrosis are a good indicator.

Listeria monocytogenes is the cause of a rare form of meningitis and may cause septicemia in humans. Focal necrosis with macrophages which contain tiny intracellular rods arranged in a 'Chinese letter' formation, and staining variably with the Gram stain, are the hallmarks of this disease.

Mycobacterium tuberculosis is the second leading cause of death from an infectious agent worldwide and remains a significant pathogen in developed countries. Disease results in the familiar caseating granulomatous lesion and its associated 1–2 μm, blunt-ended, acid- and alcohol-fast bacilli. In some regions such as Africa, tuberculosis is commonly associated with HIV infection, causing major morbidity and mortality.

Mycobacterium avium/intracellulare are representatives of a group of intracellular opportunistic mycobacteria which are frequently present in the later stages of immunosuppression, particularly associated with HIV/AIDS. They frequently persist

in spite of treatment, and are often fatal. The lesions produced are non-caseating and consist of collections of vacuolated macrophages which often contain vast numbers of organisms. On occasion, there is little evidence of a cellular reaction on an H&E-stained section, and the organism is detected only by routinely performing an acid-fast stain, such as the ZN, on all tissue from HIV/AIDS patients. This group also includes *M. kansasii*.

Mycobacterium leprae is an obligate intracellular, neurotrophic mycobacterium which attacks and destroys nerves, especially in the skin. The tissue reaction to leprosy depends on the immune status of the host. It can be minimal with a few macrophages packed with crescentic, pointed, intracytoplasmic bacilli (lepromatous leprosy), or may contain scanty organisms and show florid granulomatous response (tuberculoid leprosy). *M. leprae* is only acid-fast and is best demonstrated with the modifed Fite method (see page 261).

Legionella pneumophila was first identified in 1977 as the cause of a sporadic type of pneumonia with high mortality. The small Gram-negative coccobacillus is generally spread in aerosols from stagnant water reservoirs, usually in air-conditioning units. The bacterium may be difficult to stain except with the Dieterle and modified Steiner silver stains, and specific antiserum.

Treponema pallidum is the organism causing syphilis, and is infrequently seen in biopsy specimens when the primary lesion or 'chancre' is diagnosed clinically. The spirochete is quite obvious using dark-ground microscopy as an 8–13 µm corkscrew shaped microorganism which often kinks in the center (Fig. 16.2). Dieterle, Warthin-Starry or modified Steiner methods may demonstrate the organism. In addition, a specific antiserum is also available.

Leptospira interrogans is a spirochete organism causing leptospirosis or Weil's disease. The disease is spread in the urine of rats and dogs, causing fever, profound jaundice, and sometimes death. Spirochetes can be seen in the acute stages of the disease where they appear in Warthin-Starry and modified Steiner techniques as tightly wound 13 µm microorganisms with curled ends resembling a shepherd's crook.

Intestinal spirochetosis appears as a massive infestation on the luminal border of the colon by the spirochete *Brachyspira aalborgi* (Tomkins et al., 1986). It measures 2–6 µm long, is tightly coiled and arranged perpendicularly to the luminal surface of the gut, giving it a fuzzy hematoxyphilic coat in an H&E stain. There is no cellular response to the presence of this spirochete. It is seen well with the Warthin-Starry and the modified Steiner techniques.

Cat-scratch disease presents as a self-limiting, local, single lymphadenopathy appearing about 2 weeks after a cat scratch or bite. Histologically the node shows focal necrosis or micro-abscesses. Two Gram-negative bacteria, ***Afipia felis*** and ***Bartonella henselae*** have been implicated. It is difficult to demonstrate on paraffin sections because of the timing or maturation factor of the bacterium, but the modified Steiner and the Warthin-Starry methods are valuable techniques for demonstrating this organism.

Fungal infections

Fungi are widespread in nature, and humans are regularly exposed to the spores from many species. The most commonly encountered fungal diseases are the superficial mycoses which affect the subcutaneous or horny layers of the skin or hair shafts, and cause conditions such as athlete's foot or ringworm. These dermatophytic fungi belong to the Microsporum, Trichophyton and Epidermophyton groups and may appear as yeasts or mycelial forms within the keratin. They are seen quite well in the H&E stain, but are demonstrated better with the Grocott and PAS stains. As with other infections, the increase in the number of patients with diminished or compromised immune systems has increased the incidence of *systemic mycoses*, representing opportunistic attacks by fungi, frequently of low virulence, but often fatal if untreated.

When fungi grow in tissue they may display primitive asexual (imperfect) forms which appear as either spherical *yeast* or *spore* forms. Some may produce vegetative growth which appears as tubular *hyphae* which may be septate and branching. These features are important morphologically for identifying different types of fungi. A mass of interwoven hyphae is called a fungal *mycelium*. Only rarely, when the fungus reaches an open cavity, the body surface, or a luminal surface such as the bronchus, are the spore-forming fruiting bodies called *sporangia* or *conidia*, produced.

Identification of fungi

Some fungi may elicit a range of host reactions from exudative, necrotizing to granulomatous whereas other fungi produce little cellular response to indicate their presence. Fortunately, most fungi are relatively large, and their cell walls are rich in polysaccharides which can be converted by oxidation to dialdehydes and thus detected with Schiff's reagent or hexamine-silver stains. Fungi are often weakly hematoxyphilic and can be suspected on H&E stains. Some fungi, e.g. sporothrix, may be surrounded by a stellate, strongly eosinophilic, refractile Splendore-Hoeppli precipitate of host immunoglobulin and degraded eosinophils.

Fluorochrome-labeled specific antibodies to many fungi are available, and are in use in mycology laboratories for the identification of fungi on fresh and paraffin wax sections. These antibodies have not found widespread use on fixed tissue, where identification still relies primarily on traditional staining methods.

An H&E stain, a Grocott methenamine (hexamine)-silver (GMS), a mounted unstained section to look for pigmentation and a good color atlas (Chandler et al., 1980) when experience fails, permit most fungal infections to be identified sufficiently for diagnoses. However, there is no substitute for microbiological culture.

Grocott methenamine (hexamine)-silver for fungi and Pneumocystis species (Gomori, 1946; Grocott, 1955; Swisher & Chandler, 1982)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

4% chromic acid commercially available, or

Chromic acid	4.0 g
Distilled water	100 ml

1% sodium bisulfite

Sodium bisulfite	1 g
Distilled water	100 ml

5% sodium thiosulfate

Sodium thiosulfate	5.0 g
Distilled water	100 ml

0.21% silver nitrate, stock solution a

Silver nitrate	2.1 g
Distilled water	1000 ml

Refrigerate for up to 3 months.

Methenamine-sodium borate, stock solution b

Methenamine	27 g
Sodium borate decahydrate (borax)	3.8 g
Distilled water	1000 ml

Refrigerate for up to 3 months.

Methenamine-silver sodium borate working solution

Equal parts of solutions **a** and **b**.

Make fresh each time and filter before use.

0.2% light green stock solution

Light green	0.2 g
Distilled water	100 ml
Glacial acetic acid	0.2 ml

Light green working solution

0.2% light green stock solution	10 ml
Distilled water	50 ml

Prepare working solution fresh before each use.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Oxidize in 4% aqueous chromic acid (chromium trioxide) for 30 minutes.
3. Wash briefly in distilled water.
4. Dip briefly in 1% sodium bisulfite.
5. Wash well in distilled water
6. Place in preheated (56–60°C water bath) working silver solution for 15–20 minutes. Check control after 15 minutes. If section is 'paper bag brown' then rinse in distilled water and check under microscope. If it is not ready, dip again in distilled water and return to silver. Elastin should not be black. Check every 2 minutes from that point onwards (see Note a).
7. Rinse well in distilled water.
8. Tone in 0.1% gold chloride for 5 seconds. Rinse in distilled water.
9. Place in 5% sodium thiosulfate for 5 seconds.
10. Rinse well in running tap water.
11. Counterstain in working light green solution until a medium green (usually 5–15 seconds).
12. Dehydrate, clear and mount.

Results

Fungi, Pneumocystis, melanin	black
Hyphae and yeast-form cells	sharply delineated in black of fungi
Mucins and glycogen	taupe to dark gray/ brown
Background	pale green

Notes

a. Incubation time is variable and depends on the type and duration of fixation, and organism being demonstrated. Impregnation is controlled microscopically until fungi are dark brown. Background is colorless at this point. Over-incubation produces intense staining of elastin and fungi which may obscure fine internal detail of the hyphal septa. This detail is essential for critical identification, and is best seen on under-impregnated sections. To avoid excess glycogen impregnation in liver sections, section may be digested prior to incubation. A water bath may be used effectively to insure an even incubation temperature.

b. Borax ensures an alkaline pH.

c. Sodium bisulfite removes excess chromic acid.

d. Some workers prefer a light H&E counterstain. This is especially useful when a consulting case is sent with only one slide, providing morphological detail for the pathologist.

e. Solutions **a** and **b** need to be made and stored in chemically clean glassware (20% nitric acid), as does the working solution. This includes graduates and Coplin jars. Do not use metal forceps.

f. Allow all refrigerated solutions to reach room temperature before using.

McManus' PAS method for glycogen and fungal cell walls

Fixation

10% NBF.

Sections

3–5-µm paraffin wax sections.

Solutions

Schiff's reagent, see page 183 or commercially available.

0.5% periodic acid solution

Periodic acid	0.5 g
Distilled water	100 ml

0.2% light green stock solution

Light green	0.2 g
Distilled water	100 ml
Glacial acetic acid	0.2 ml

This is the same stock solution used in the GMS.

Light green working solution

0.2% light green stock solution	10 ml
Distilled water	50 ml

Make fresh before each use.

Method

1. Deparaffinize and hydrate slides to distilled water.
2. Oxidize in periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Place in Schiff's reagent for 15 minutes.
5. Wash in running tap water for 10 minutes to allow pink color to develop.
6. Counterstain for a few seconds in working light green solution.
7. Dehydrate in 95% alcohol, absolute alcohol and clear in xylene.
8. Mount in resin-based mountant.

Results

Fungal cell walls and glycogen	magenta to red
Background	pale green

Note

A solution of 5% aqueous sodium hypochlorite reduces overstaining by Schiff's.

A selection of the more important fungi and actinomycetes

Actinomyces israelii is a colonial bacterium which can be found as a commensal in the mouth and tonsillar crypts. It can cause a chronic suppurative infection, actinomycosis, which is characterized by multiple abscesses drained by sinus tracts. Actinomycotic abscesses can be found in the liver, appendix, lung and neck. The individual organisms are Gram-positive, hematoxyphilic, non-acid-fast, branching filaments 1 µm in diameter. They become coated in 'clubs' of Splendore-Hoeppli protein when the organism is invasive. These clubs are eosinophilic, acid-fast, 1–15 µm wide and up to 100 µm long, and stain

polyclonally for immunoglobulins. This arrangement of a clump of actinomyces or fungal-like hyphae, which measures 30–3000 µm, surrounded by eosinophilic protein, is called a 'sulfur' granule and is also an important identification marker for other fungal groups. These granules may be macroscopically visible and their yellow color is an important diagnostic aid.

Nocardia asteroides is another actinomycete. It is filamentous and may be visible in an H&E stain, but it is Grocott-positive and variably acid-fast using the modified ZN for leprosy. However, it is difficult to demonstrate even with the acid-fast bacillus techniques. Its pathology is similar to that of actinomycosis, but its organisms are generally more disseminated and it tends to cause invasive infection in the immunocompromised.

Candida albicans is a common yeast, but with immunosuppression may become systemic. It infects the mouth (thrush), the esophagus, the vagina (vaginal moniliasis), the skin and nails, and may be found in heart-valve vegetations. It is seen as both ovoid budding yeast-form cells of 3–4 µm, and more commonly as slender 3–5 µm, sparsely septate, non-branching hyphae and pseudo-hyphae. Whilst difficult to see on H&E, this organism is strongly Gram-positive, and is obvious with the Grocott and PAS techniques.

Aspergillus fumigatus is a soil saprophyte and a commensal in the bronchial tree. It may infect old lung cavities (Fig. 16.3) or become systemic in immunosuppressed patients. The fungus has broad, 3–6 µm, parallel-sided, septate hyphae showing dichotomous (45 degree) branching. It may be associated with Splendore-Hoeppli protein and sometimes forms fungal balls within tissue. This fungus may be seen in an H&E stain and is demonstrated well with a PAS or Grocott. When it grows exposed to air, the conidophoric fruiting body may be seen as *Aspergillus niger*, a black species which can cause infection of the ear.

Zygomycosis is an infrequently seen disease caused by a group of hyphated fungi belonging mainly to the genera Mucor and Rhizopus. They have thin-walled hyphae (infrequently septate) with non-parallel sides, ranging from 3 to 25 µm in diameter,

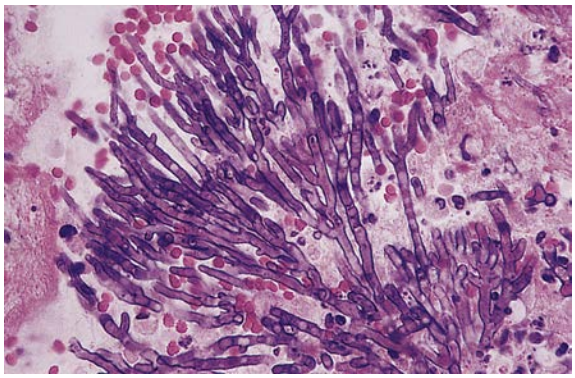


Fig. 16.3 A strong hematoxylin (Ehrlich's) and eosin stain will show the fine detail of many infectious agents. The hyphal structure identifies this as *Aspergillus fumigatus* which was colonizing an old tuberculosis cavity in the lung.

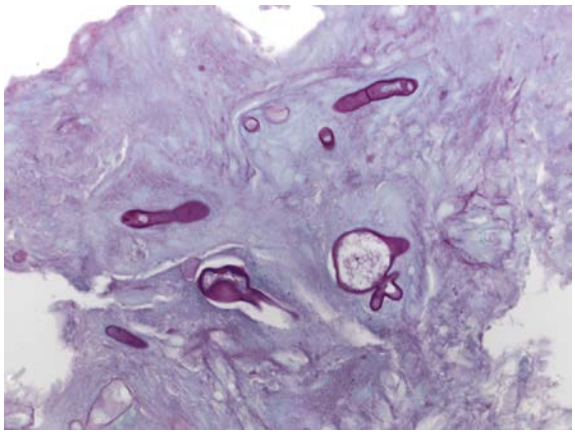


Fig. 16.4 Rhizomucor (a cosmopolitan, filamentous fungus) is well demonstrated by this PAS stain with light green counterstain.

branch irregularly, and often show empty bulbous hyphal swelling. Grocott and PAS are the staining methods of choice (Figs. 16.4 and 16.5).

Cryptococcus neoformans exists solely in yeast-form cells, is variable in diameter, 2–20 µm, with ovoid, elliptical and crescentic forms frequently seen. There is an extensive mucopolysaccharide coat around the yeasts which is mostly dissolved during processing, but when present, appears as a halo around the organism and is visible with special stains such as Mayer's or Southgate's mucicarmine procedures. Yeasts may be free form or within the

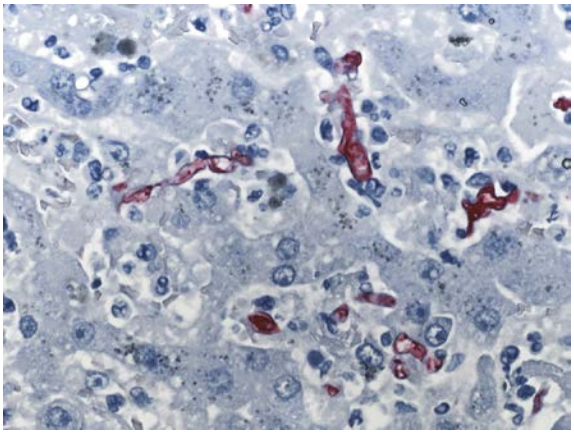


Fig. 16.5 Immunohistochemistry is being increasingly applied to the demonstration of microorganisms using labeled specific antibody. This figure demonstrates Zygomycetes, a fast-growing fungus, with fast red chromogen.

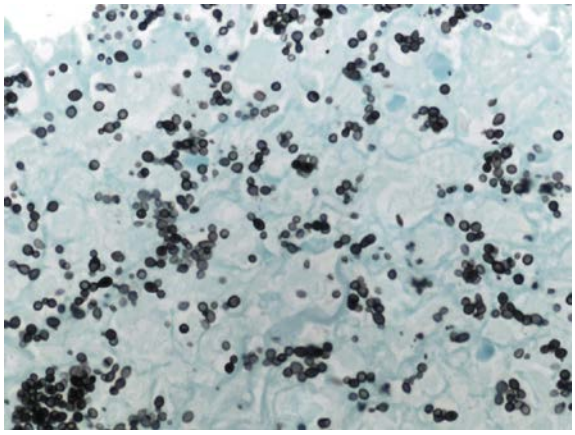


Fig. 16.6 Grocott's methenamine-silver stains a wide variety of infectious agents. Here seen with light green counterstain is the method of choice for *Histoplasma capsulatum*, a dimorphic endemic fungus.

cytoplasm of giant cells, staining faintly with an H&E stain. The PAS and Grocott procedures demonstrate these cells well. Infection is found in the lungs and in the brain within the parenchyma or in the leptomeninges. Often, these patients are immunosuppressed.

Histoplasma capsulatum is another soil-dwelling yeast which can cause a systemic infection in humans called histoplasmosis. It is especially common along the southern border of the United States, and where there are large bird populations. The organism is usually seen within the cytoplasm of macrophages which appear stuffed with small, regular, 2–5 µm yeast-form cells which have a thin halo around them in H&E and Giemsa stains. Langhans' giant cells, forming non-caseating granulomas may be present. PAS and Grocott stains demonstrate this fungus well (Fig. 16.6).

Pneumocystis jiroveci. There is still some debate over the taxonomy of this organism, although analysis of its ribosomal RNA has placed it nearer to a fungal than a protozoan classification (Edman et al., 1988). It came to prominence as a pathogen following immunosuppressive therapies associated with renal transplants in the 1960s, and has become a life-threatening complication of HIV. It most frequently causes pneumonia, where the lung alveoli are progressively filled with amphophilic, foamy plugs of parasites and cellular debris. It is found rarely in other sites such as the intestines and lymph nodes. The cysts are invisible in an H&E stain, and can barely be seen in a Papanicolaou stain as they appear refractile when the microscope condenser is racked down. Specific immunohistochemistry is available to use, otherwise, Grocott methenamine-silver is recommended. Only electron microscopy or an H&E stain on a resin-embedded thin section will show their internal structure. The cysts are 4–6 µm in diameter and contain 5–8 dot-like intracystic bodies. The cysts rupture and collapse, liberating the trophozoites which can be seen as small hematoxyphilic dots in a good H&E and Giemsa stain; these attach to the alveolar epithelium by surface philopodia.

The demonstration of rickettsia

Rickettsial organisms, e.g. those causing Q fever, Rocky Mountain spotted fever or typhus, rarely need to be demonstrated in tissue sections. They can sometimes be seen with a Giemsa stain, or by using the Macchiavello technique which also demonstrates some viral inclusion bodies (Fig. 16.7).

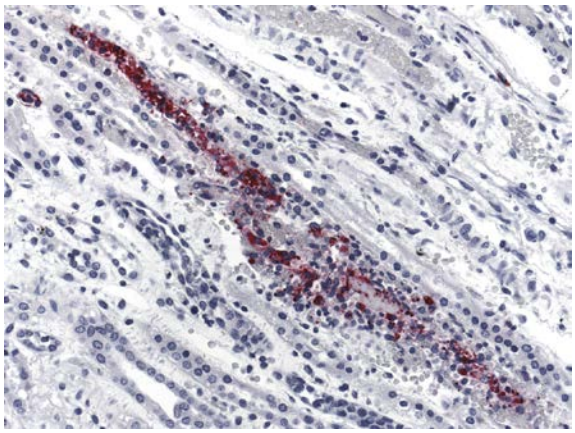


Fig. 16.7 Immunohistochemical method demonstrating Rocky Mountain spotted fever in kidney. It is caused by the bacterium *Rickettsia rickettsii*, which is carried by ticks.

Macchiavello's stain for rickettsia and viral inclusions (modified Culling, 1974)

Sections

Formalin fixed, paraffin wax embedded.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain in 0.25% basic fuchsin for 30 minutes.
3. Differentiate in 0.5% citric acid for 3 seconds.
4. Wash in tap water for 2 minutes.
5. Counterstain in 1% methylene blue for 15–30 seconds.
6. Rinse in tap water.
7. Dehydrate, clear, and mount.

Results

Rickettsia and some viral inclusions	red
Background	blue

The detection and identification of viruses

Whilst the cytopathic effects of viruses can often be seen in a good H&E stain, and may be characteristic of a single viral group, the individual viral particles are too small to be seen with the light microscope, and require an electron microscope to reveal their structure. This may allow a rapid and accurate diagnosis in viral infections. Some viruses aggregate within cells to produce *viral inclusion bodies* which may be intranuclear, intracytoplasmic or both. These inclusion bodies may be acidophilic and usually intranuclear, or can be basophilic and cytoplasmic. Most special staining methods are modified trichromes using contrasting acid and basic dyes to exploit these differences in charges on the inclusion body and the host cell. These methods include Mann's methyl blue-eosin stain for the Negri bodies of rabies, Macchiavello's method, and more recently the elegant Lendrum's phloxine-tartrazine stain. Unfortunately, the need for optical differentiation in these methods increases the chance of technical error.

The introduction of commercially available monoclonal immunohistology to viruses, which are either class or species specific, has revolutionized the tissue detection of viruses. Hepatitis B virus is a good example of the diagnostic value of this technique where the surface antigen (also known as HBs or Australia antigen) and the core antigen (HBc) can be specifically detected immunohistochemically, providing clinically important information about the stage of the disease. More recently, nucleic acid hybridization probes and PCR testing have become available and can be used to detect genomically inserted viral nucleic acid in situ, in cells and tissues that are frozen or formalin fixed. It should be remembered, however, that the detection of microorganisms using nucleic acid probes, unlike specific biotinylated antiserum, does not necessarily mean active disease.

Phloxine-tartrazine technique for viral inclusions (Lendrum, 1947)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

Phloxine

Phloxine	0.5 g
Calcium chloride	0.5 g
Distilled water	100 ml

Tartrazine

A saturated solution of tartrazine in 2-ethoxyethanol, or cellosolve.

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Stain nuclei in alum hematoxylin (Carazzi's or Harris's) for 10 minutes.
3. Wash in running tap water for 5 minutes.
4. Stain in phloxine solution for 20 minutes.
5. Rinse in tap water and blot dry.
6. Controlling with the microscope, stain in tartrazine until only the viral inclusions remain strongly red, 5–10 minutes on average.
7. Rinse in 95% alcohol.
8. Dehydrate, clear, and mount.

Results

Viral inclusions	bright red
Red blood cells	variably orange/red
Nuclei	blue/gray
Background	yellow

Notes

All tissue is stained red with phloxine, which is then differentiated by displacement with the counter-stain tartrazine. The red color is first removed from muscle, then other connective tissues. Paneth cells, Russell bodies, and keratin can be almost as dye retentive as viral inclusions, and can occasionally be a source of confusion.

Shikata's orcein method for hepatitis B surface antigen (modified Shikata et al., 1974)

Sections

Formalin fixed, paraffin wax embedded.

Solutions

Acid permanganate

0.25% potassium permanganate	95 ml
3% aqueous sulfuric acid	5 ml

Orcein

Orcein (synthetic)	1 g
70% alcohol	100 ml
Concentrated hydrochloric acid (gives a pH of 1–2)	1 ml

Tartrazine solution

Saturated tartrazine in cellosolve (2 ethoxyethanol)

Method

1. Deparaffinize and rehydrate through graded alcohols to distilled water.
2. Treat with acid permanganate solution for 5 minutes.
3. Bleach until colorless with 1.5% aqueous oxalic acid for 30 seconds.
4. Wash in distilled water for 5 minutes, then in 70% alcohol.
5. Stain in orcein solution at room temperature for 4 hours, or in a Coplin jar of 37°C preheated orcein for 90 minutes.
6. Rinse in distilled alcohol and examine microscopically to determine desired staining intensity.
7. Rinse in cellosolve, stain in tartrazine for 2 minutes.
8. Rinse in cellosolve, clear and mount.

Results

Hepatitis B infected cells, elastic and some mucins	brown/black
Background	yellow

Notes

The success of this method largely depends on the particular batch of orcein used, and on freshly prepared solutions. This method relies on permanganate oxidizing of sulfur-containing proteins to sulfonate residues which react with orcein. Results compare well with those obtained using labeled antibodies, but the selectivity is inferior.

Viral infections

Whilst not exhaustive, this brief summary reflects some viruses which are encountered in surgical and post-mortem histopathology (Table 16.3).

Viral hepatitis is caused by a number of viral infections. The hepatitis viruses (HV) A, B, C, D, and E show great biological diversity and are clinically the most common viruses which primarily affect the liver. The liver is the target organ and damage varies with the viral strain, ranging from massive acute necrosis to chronic 'piecemeal necrosis' of liver cells, leading to cirrhosis. An eosinophilic 'ground glass' appearance is seen in the cytoplasm of some hepatocytes due to dilated smooth endoplasmic reticulum which contains tubular HB surface antigen. It is this component which can be demonstrated using Shikata's orcein method, or by specific immunohistochemistry.

Table 16.3 Viral infections seen in histopathology

Virus	Family	Genome	Disease
Measles	Paramyxo	SS RNA	Measles
Varicella-zoster	Herpes	DS DNA	Chickenpox, shingles
Herpes simplex	Herpes	DS DNA	Cold sores, genital herpes
Cytomegalovirus (CMV)	Herpes	DS DNA	Cytomegalic inclusion disease
Epstein-Barr virus	Herpes	DS DNA	Glandular fever, African Burkitt's lymphoma
Human T-cell leukemia virus (HTLV-1)	Retro	SS RNA	Adult T-cell leukemia
Human immunodeficiency virus (HIV)	Retro	SS RNA	AIDS
Human papilloma viruses (HPV)	Papova	DS DNA	Human wart viruses
JC virus	Papova	DS DNA	Progressive multifocal leukoencephalopathy (PML)
Poliovirus	Picorna	SS DNA	Poliomyelitis
Molluscum virus	Pox	DS DNA	Molluscum contagiosum
Lyssavirus	Rhabdo	SS RNA	Rabies

DS = double-stranded; SS = single-stranded.

Herpes viruses are usually acquired subclinically during early life and enter a latent phase, to be reactivated during times of immunological stress. These viruses cause blistering or ulceration of the skin and mucous membranes, but can cause systemic diseases, including encephalitis, in immunosuppressed or malnourished individuals. The cytopathic effects of the herpes virus are well seen in Tzanck smears of blister fluid, and include the margination of chromatin along nuclear membranes, Cowdry type A ('owl's eye') inclusion bodies, and syncytial ('grape-like') nuclei within giant cells.

Cytomegalovirus (CMV) causes congenital infections of newborns and can result in systemic disease in HIV and immunosuppressed patients. It is seen in the endothelial cells forming prominent intranuclear inclusions which spill into the cytoplasm where they form granular hematoxyphilic clusters. The CMV virus causes obvious cytomegaly in the cells it infects. All herpes viruses have an identical electron microscopic appearance of spherical, 120 nm, membrane-coated particles.

Papilloma viruses are a family of about 50 wart viruses which cause raised verrucous or papillomatous skin warts, or flat condylomatous genital warts. Cytologically, evidence of hyperkeratosis may be present together with koilocytosis (irregular nuclear enlargement and cytoplasmic vacuolation forming perinuclear halos). Skin verrucas are associated with HPV 1–4 strains, genital condylomas with HPV 6, 11, 16 and 18, and cervical cancer with HPV 16 and 18. These uncoated viruses measure 55 nm, are mainly intranuclear, and can be detected using electron microscopy, or immunoperoxidase and gene probes on paraffin wax sections.

JC (John Cunningham) ***virus*** is a polyomavirus (previously known as papovavirus) which causes progressive multifocal leukoencephalopathy, a demyelinating disease, in HIV and other immunosuppressed patients. Intranuclear hematoxyphilic inclusions may be seen within swollen oligodendrocytes.

Molluscum virus produces a contagious wart in children and young adults called molluscum contagiosum. Large eosinophilic, intracytoplasmic inclusion bodies can be seen in maturing keratinocytes on routine H&E sections, and are seen well with phloxine-tartrazine. The large 1 μm viral particles

have a typical pox virus structure: brick-shaped with a superimposed figure-of-eight nucleic acid sequence.

Rabies virus. This neurotrophic rhabdovirus forms intracytoplasmic eosinophilic inclusions best seen in the axonal hillocks of hippocampal neurons of the brain. Macchiavello, phloxine-tartrazine, Mann's methyl blue-eosin or PAS stains are recommended. Note that given the pathogenicity of these agents, if suspected, the case should be passed to a relevant diagnostic center rather than a routine laboratory.

Human immunodeficiency virus (HIV) consists of at least two retrovirus strains. The virus is best seen in cultured lymphocytes and is rarely seen in tissues from affected patients. It produces a distinctive neuropathological lesion in HIV encephalitis consisting of microglial nodules, or stars, containing collections of giant cells, microglia and astrocytes. Synthetic nucleic acid probes have been prepared to identify HIV genomes.

Influenza (flu) is a contagious respiratory illness caused by influenza viruses (Fig. 16.8). It can cause mild to severe illness, and at times can lead to death. According to the Centers for Disease Control (CDC) every year in the United States, on average, 5–20% of the population suffers from the flu, more than 200,000 people are hospitalized from flu complications, and about 36,000 people die from flu. More recently, concern about the influenza A H5N1 strain of bird flu has emerged. Some people, e.g. older people, young children and people with certain health conditions, are at high risk of serious flu complications.

SARS (severe acute respiratory syndrome) and ***MERS*** (Middle East respiratory syndrome) can be severe respiratory illnesses caused by a coronavirus (SARS-CoV and MERS-CoV) (Fig. 16.9). SARS was first reported in Asia in February 2003. Over the next few months, the illness spread to more than two dozen countries in North America, South America, Europe and Asia before the SARS global outbreak of 2003 was contained. MERS-CoV is a novel coronavirus, first identified in Saudi Arabia in 2012 and the majority of cases emerging from the Arabian Peninsula. The risk of infection is linked to exposure to camels and subsequent outbreaks have occurred in healthcare facilities. The incidence and spread of MERS remains an ongoing matter of global public health importance.

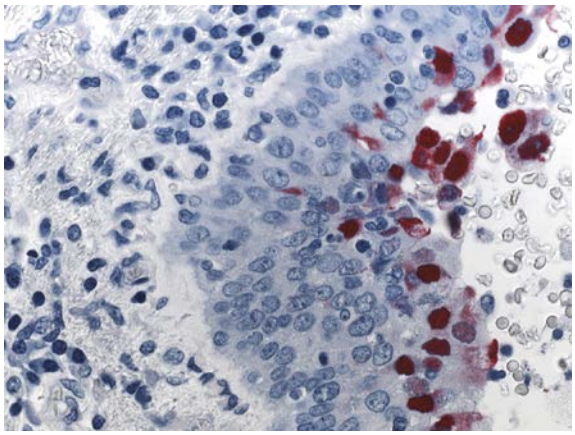


Fig. 16.8 Immunohistochemical method demonstrating influenza A virus-infected cells in the bronchus.

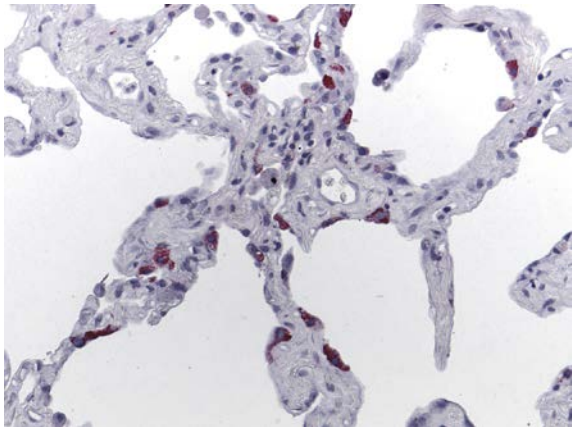


Fig. 16.9 Immunohistochemical method demonstrating the previously unrecognized SARS-associated coronavirus which is responsible for severe acute respiratory syndrome (SARS).

Prion disease

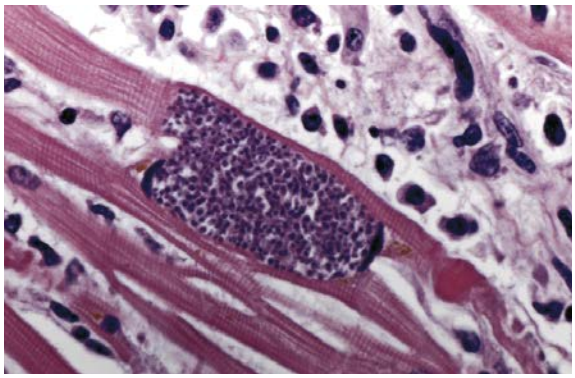
To date, more than eight transmissible neurodegenerative diseases have been described affecting the central nervous system (CNS). The diseases caused by prions include Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD), Germann-Straussler-Shienker

disease, fatal familial insomnia and kuru in humans and in animals, bovine spongiform encephalopathy (BSE, also known as 'mad cow disease'), scrapie (in goats and sheep), and chronic wasting disease (CWD) (in mule deer and elk). Prions are not microbes in the usual sense because they are not alive, but the illness they cause can be transmitted from one animal to another. All usually produce a characteristic spongiform change, neuronal death and astrocytosis in affected brains. The infectious agent is a prion, a small peptide, free of nucleic acid and part of a normal transmembrane glycoprotein which is not, strictly speaking, a virus. Antibodies have been prepared from prion protein which strongly mark accumulated abnormal protein in these diseases (Lantos, 1992).

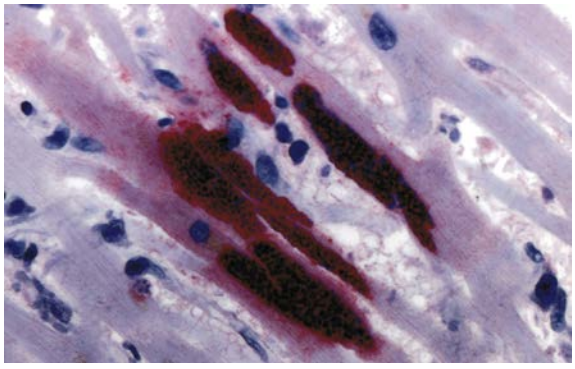
The CJD Surveillance Center in the USA is an invaluable source for monitoring and testing human prion disease in the United States. The Center is supported by the CDC and by the American Association of Neuropathologists. Visit their website (http://www.cjdsurveillance.com) for details on how to submit specimens for testing as they perform these tests at no charge for laboratories in the USA. In addition, both CDC and the World Health Organization (WHO) also offer guidelines regarding the handling of suspected and known cases of prion disease. Visit http://www.cdc.gov and search for CJD for a fact sheet and other relevant information. WHO offers a manual in pdf form for downloading. It gives information about what to do should you find yourself with a suspected or known positive case in your laboratory: http://who.int/bloodproducts/TSE-manual2003.pdf. Remember that these types of cases should never knowingly be handled in a routine histology laboratory. Contact your local health department for additional guidelines.

The demonstration of protozoa and other organisms

The identification of protozoa is most often made on morphological appearance using H&E and, particularly, Giemsa stains. The availability of antisera against organisms such as entamoeba, toxoplasma, and leishmania has made diagnosis much easier in difficult cases (Fig. 16.10a,b).



a



b

Fig. 16.10 (**a**) H&E and (**b**) immunohistochemical methods demonstrating the single-celled parasite *Toxoplasma gondii* in heart.

Giemsa stain for parasites

Sections

Fixative is not critical, but B5 or Zenker's is preferred; thin, 3 µm paraffin wax sections (if Zenker's is not used, post-mordant in Zenker's in a 60°C oven for 1 hour before staining).

Solutions

Giemsa stock solution, commercially available or

Giemsa stain powder	4 g
Glycerol	250 ml
Methanol	250 ml

Dissolve powder in glycerol at 60°C with regular shaking. Add methanol, shake the mixture.

Working Giemsa solution for parasites

Giemsa stock	4 ml
Acetate buffered distilled water, pH 6.8	96 ml

Method

1. Deparaffinize and rehydrate through graded alcohols to water.
2. Rinse in pH 6.8 buffered distilled water.
3. Stain in working Giemsa, overnight.
4. Rinse in distilled water.
5. Rinse in 0.5% aqueous acetic acid until section is pink.
6. Wash in tap water.
7. Blot until almost dry.
8. Dehydrate rapidly through alcohols, clear and mount.

Results

Protozoa and some other microorganisms	dark blue
Background	pink/pale blue
Nuclei	blue

Protozoa

Entamoeba histolytica, the organism causing amebic colitis or dysentery, can be found in ulcers which occur in infected colon and in amebic liver abscesses. The trophozoite (adult form) measures 15–50 µm, contains a small nucleus and has a foamy cytoplasm containing ingested red cells and white cell debris. They may be seen in granulation tissue within ulcers on routine H&E staining, or in the luminal mucus overlying normal-appearing mucosa. They are PAS-positive and brief counterstaining in 1% aqueous metanil yellow emphasizes the ingested red cells.

Toxoplasma gondii, a commonly encountered organism which is spread in cat litter, causes an acute lymphadenopathy which is often subclinical. Affected nodes show non-specific changes and no organisms. In AIDS and other immunosuppressed patients this protozoon causes systemic diseases, including meningoencephalitis where encysted bradyzoites and free tachyzoites can be seen in necrotic brain tissue. Cysts also occur in other tissues such as cardiac muscle, and measure up to 40 µm with tachyzoites (4–6 µm), which can be seen on H&E. A Giemsa stain can also be used, but the use of labeled specific antiserum is recommended (Fig. 16.10).

Leishmania tropica is transmitted by sand-fly bites and causes a chronic inflammatory disease of the skin sometimes called cutaneous leishmaniasis. The injected parasite forms (2 µm), or amastigotes, are found in large numbers within the cytoplasm of multiple swollen histiocytes which congregate in early lesions in the dermis. A related organism, *L. donovani*, causes a systemic visceral infection, kala azar, in which the organisms are seen within histiocytes in the spleen, lymph nodes, liver and bone marrow. The organisms are hematoxyphilic and can be emphasized with a Giemsa stain.

Giardia duodenalis (lamblia) is a flagellate protozoon which is ingested in cyst form from drinking water with fecal contamination. The trophozoites migrate to the duodenum where they may cause severe diarrhea and malabsorption. These organisms have been seen on an H&E stain where they appear as eosinophilic, sickle-shaped flakes with indistinct nuclei, resting on intestinal mucosa which may show little evidence of inflammation. When seen in a fresh Giemsa-stained duodenal aspirate, they appear kite-shaped, 11–18 µm in size, binucleate and have faint terminal flagella.

Trichomonas vaginalis is a similar flagellate protozoon most frequently seen in a Papanicolaou stain. Inflammatory cells and mildly dysplastic squamous cells often accompany this parasite as it causes cervicitis in the female, and urethritis in both sexes.

Cryptosporidium is one of a group of protozoa (including Isospora and Microsporidium) which causes severe and relentless outbreaks of diarrhea among HIV patients. Cryptosporidial gametes are seen on H&E stain as blue dots arranged along the mucosal surface. Mature cysts are shed into feces and are acid-fast in a ZN stain of fecal smears.

Worms

Schistosoma species cause the disease schistosomiasis or 'bilharzia'. Various manifestations of the disease differ according to the particular *Schistosoma* species involved, but granulomata containing schistosome ova are found in the liver, bowel and bladder mucosa, and occasionally in the lungs. The ova have thick, refractile, eosinophilic walls and are easily

detected in H&E-stained sections. The PAS, Grocott and ZN techniques are positive for these ova. Where the plane of section allows, the presence of a terminal spine to the ovum indicates *S. haematobium*, whereas *S. mansoni* and *S. japonicum* have lateral spines. Any good trichrome procedure will demonstrate worm development.

Echinococcosis. *Echinococcus granulosus* is a tapeworm found in dogs, but humans and sheep may become intermediate hosts and develop hydatid cyst disease. These cysts form in many organs, particularly liver and lung. The walls of the daughter cysts are faintly eosinophilic, characteristically laminated, and produced by the worm, not by its host. The walls are PAS-positive and Congo red-positive, showing green birefringence. The scolicial hooklets survive inside old, burnt-out cysts, they have a diagnostic shape and stain brilliant yellow with picric acid.

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17 Bone

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Introduction

Bone is considered the most important supporting tissue in the body. It is composed of cells, organic extracellular matrix and inorganic salts. Bone tissue is mineralized in layers which provide great strength and flexibility to the skeletal system. It varies in formation, depending on its function, across the body. These functional/formation differences are also based on the proportion of the different inorganic and organic processes incorporated or produced in the formation of a bone. The most common mineral in bone is hydroxyapatite which consists of collagen, proteins and carbonate ions. The main bulk of bone is approximately 70% mineral and 30% organic components by weight. Bone cells, as opposed to marrow cells, are relatively sparse. This chapter will review bone morphology and its organic and inorganic components, with a focus on problematic microtomy, as well as considering methods on preparing sections of bone for analysis which can be used in clinical and research histology laboratories.

Normal bone

Two types of bone can be recognized macroscopically in the adult human skeleton. These are cortical or compact bone and trabecular, cancellous, or spongy bone. Compact bone is the solid, hard and immensely strong tissue which forms the shafts of long bones, e.g. femur and tibia, and exterior surfaces of the flat bones, e.g. ribs and skull. Trabecular bone is found in the diaphysis, epiphysis and marrow cavities of long bones, vertebrae and the centers of flat bones. The latter is a mesh of bone strands each about 1 mm thick. Although it looks less solid than cortical bone, this arrangement of trabeculae, particularly in the femoral head and vertebrae forms an almost ideal weight-bearing structure, and is strong.

The major components of bone are mineral, cells and an organic extracellular matrix of collagen fibers and ground substance. These are dynamic components, as the processes of cell replacement, repair and remodeling of bone, and the erosion and reformation of collagen and mineral occur continually throughout adult life.

Bone collagen

The collagen found in bone differs from other collagen in the body as it is mineralized, and laid down in bands or lamellae roughly parallel to one another. The collagen fibers within each lamella tend to lie next to each other but at an angle to the fibers in adjacent lamellae. A cement of proteoglycan ground substance outlining these fibers is seen in sections only at the cement lines. The organization of collagen lamellae is responsible for the distinctive microanatomical patterns of bone which are easily seen with polarized light microscopy. The simplest pattern occurs on the periosteal and endosteal surfaces of compact bone as circumferential lamellae, and in trabecular or non-Haversian bone where lamellae are roughly parallel to the surface. Cortical bone is composed of Haversian systems or osteons in which concentric lamellae surround channels (Volkmann's canals) containing one or more blood vessel. These tubular structures run longitudinally in the bone and are packed closely together with irregular interstices filled by the remnants of older osteons (Fig. 17.1).

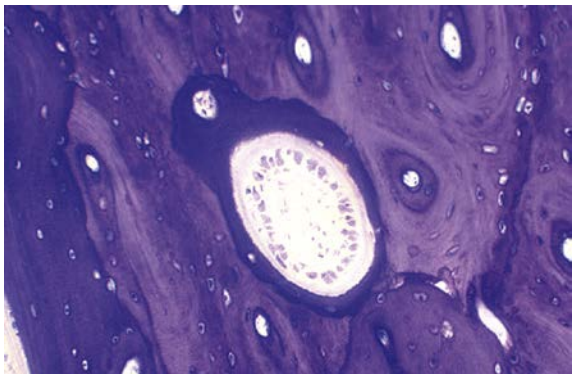


Fig. 17.1 Haversian systems (osteons, Volkmann's canals and bone cells) in ground section of undecalcified bone in methyl methacrylate, acid etched/surfaced and stained with McNeal's/toluidine blue stain.

Cement lines outline the boundaries of osteons, and some trabecular and circumferential lamellae.

Another collagen fiber arrangement forms non-lamellar or woven bone and is found in immature bone and some pathological conditions. This collagen is not deposited in the lamellae but in thick, short, randomly oriented bundles. When viewed with polarized light, these appear as coarse fibers resembling a woven fabric.

Unmineralized collagen or osteoid forms a border, or seam, on surfaces of newly formed bone, and after osteoid is deposited there is a lag before it becomes mineralized. Osteoid is normally around 15 µm thick, covering only a small proportion of the surfaces. In some diseases, e.g. rickets or osteomalacia, it is much thicker and widespread.

On inactive surfaces the osteoid is thin, difficult to see, and is completely absent where resorption is taking place. This process is called remodeling, and consists of resorption and deposition taking place in equilibrium so that the volume and shape of bones stays more or less constant. In later life, remodeling slows down, and deposition may not keep up with resorption, causing increased bone porosity and brittleness, and in extreme cases the disease osteoporosis occurs.

Bone mineral

The main mineral content of bone is calcium and phosphate combined with hydroxyl ions to form hydroxyapatite crystals. This mineral is approximately 38% calcium and thought to be deposited as amorphous calcium phosphate in the initial mineralization phase. This transforms to hydroxyapatite by the addition of hydroxyl ions to form a crystal lattice into which carbonate, citrate and fluoride ions as well as magnesium, potassium and strontium can be substituted or included. Carbonate is present in large quantities, but probably only in the hydration shell and on crystal surfaces.

The hydroxyapatite forms needle-like crystals about 22 nm in length, resulting in an enormous total crystal surface area. Thus, the mineral fulfills the obvious function of giving strength and rigidity, whilst approximately 20% remains in the amorphous form to provide a readily available buffer for maintaining total body chemical equilibrium e.g. pH and enzyme system regulation.

Bone cells

There are three types of bone cell found within bone tissue. The cells in the bone marrow belong to the hematopoietic system and will not be discussed in this chapter.

Osteoblasts

These cells are fully differentiated to carry out the primary function of bone formation by producing and laying down osteoid. They are seen on surfaces of actively forming bone as plump cells with basophilic cytoplasm and eccentric nuclei distal to the bone surface. The cytoplasm is basophilic due to its ribonucleic acid content, and before becoming fully differentiated, frequently contains glycogen. Acid phosphatase is found in osteoblasts and the surrounding tissues, but decreases at the onset of calcification. Upon completion of bone formation, most osteoblasts become quiescent, small cells residing among the heterogeneous cell population.

Osteocytes

Osteoblasts are active producers of osteoid, but become mature osteocytes after they are trapped in the lacunae of the calcified osteoid matrix. Lacunae are connected to each other and to the vascular

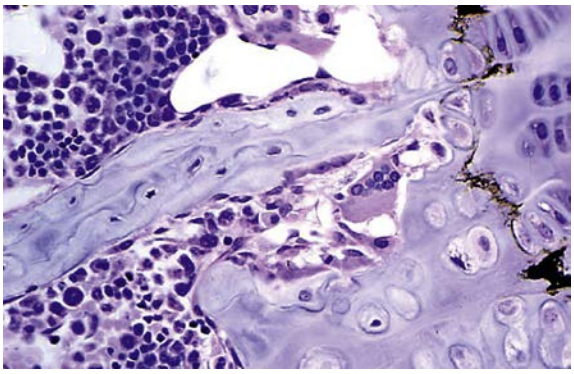


Fig. 17.2 Osteoclasts lining trabecular bone. Undecalcified rat knee in GMA microtomed at 2 µm. Hematoxylin and eosin stain.

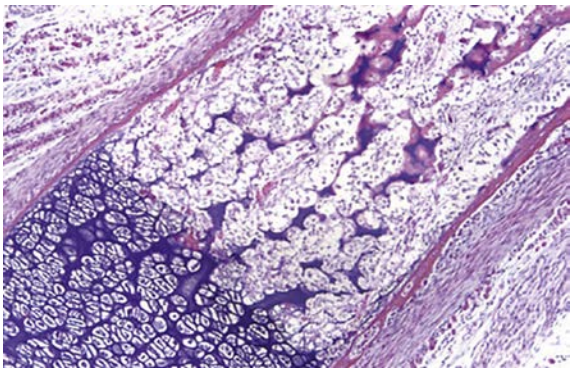


Fig. 17.3 Fetal long bone development, approximate age 57 days. Formation of trabecular bone with a high concentration of chondrocytes at the ends of the bone.

spaces by canaliculi, tiny channels into which osteocyte processes project. These processes absorb dissolved substances necessary for cell metabolism from the passing fluids.

Osteoclasts

These are the cells responsible for bone resorption or erosion. Osteoclasts are usually large, multinucleated (giant) cells whose cytoplasm contains numerous mitochondria and alkaline phosphatase. They occur in small clusters, or singly, on bone surfaces undergoing resorption and are often seen in the depressions (Howship's lacunae) they are actively creating by erosion. These surfaces have an irregular outline and lack osteoid (Fig. 17.2). The direction of resorption is random, with no relationship to lamellar structure. Osteoclasts respond to altered mechanical stresses on the skeleton and to growth, this activity contributes to remodeling. They also respond to hormones which can either stimulate or inhibit their activity. When resorption halts, the process of bone formation resumes (osteoid, mineralization, etc.). Cement lines will occur at the junction between old and newly formed bone.

Development and growth

Bone develops in two different ways according to the site and shape of the bone. It begins early in the embryo and is not complete until approximately 15 years of age.

Intramembranous ossification

This occurs in flat bones, e.g. skull, sternum and pelvic bones. A fibrous membrane first develops at the site of bone formation where mesenchymal cells differentiate into osteoblasts, beginning the bone formation process by laying down osteoid. This starts in small islets which gradually unite to become trabeculated and finally form an external layer of compact bone.

Endochondral ossification

This occurs in long bones and major parts of the skeleton. This type of bone development begins with the differentiation of mesenchymal cells at sites where bone will be formed, but this bone is laid down in a cartilage model resembling the final shape of the bone. This cartilage model becomes covered with a connective tissue sheath or perichondrium, growing both by apposition and interstitially. Appositional growth or the laying down of more cartilage begins towards the exterior and is mainly responsible for increased diameter; interstitial growth is by cells dividing within the model and mainly occurs towards the extremities, resulting in increased length (Fig. 17.3).

In the central part of the model, cells continue to differentiate, cartilage begins to calcify, blood vessels invade, and the cartilage is broken up into strands. Ossification by differentiation of perichondrial cells into osteoblasts, begins around the exterior of the primary ossification site at the center of the model. Osteoblasts invade the strands of calcified cartilage

and deposit osteoid which soon becomes calcified. This process continues and secondary ossification sites appear at each end of the model, separated from the new bony shaft by cartilage growth or epiphyseal plates capable of interstitial growth. Once bone has ossified, it can only grow by apposition. Remodeling is continuous. In some places deposition exceeds resorption, in others this is reversed resulting in the characteristic shape of the bone. When bone is fully grown, the three ossification sites unite and the cartilage growth plates disappear.

Techniques for analyzing bone

Techniques for the demonstration of bone and its components are possibly more varied and difficult than for any other tissue. They include:

- Decalcified bone for frozen, paraffin and transmission electron microscopy (TEM).
- Mineralized bone for frozen, plastic (microtomed or sawn/ground sections), TEM or scanning electron microscopy (SEM) samples.

The technique chosen for examination of bone is influenced by the initial clinical diagnosis, case urgency and the extent of the investigation required. Specimens arriving in the laboratory can vary in size from a needle biopsy a few millimeters long, to whole appendages, i.e. an amputation. Mineralized sections are used for microradiographic and histomorphometric studies as well as polarized and fluorescent light microscopy.

Biopsies

These are used for the diagnosis of several diseases, e.g. cancers, hematopoietic disorders and infections. These specimens are usually small enough to treat like soft tissue, except a bone biopsy usually needs decalcification. This is particularly necessary if it contains a piece of cortical bone producing paraffin wax sections. A bone marrow biopsy is usually removed with a Jamshidi needle for diagnosis of metabolic bone disease. Metabolic bone diseases are diagnosed using trabecular bone (Byers & Smith, 1967) taken from the iliac crest. This is an accessible bone site representative of skeletal bone as a whole.

Sections which are requisite to assess the relationship between mineralized and unmineralized bone (osteoid) are best processed and embedded into a plastic, e.g. methyl methacrylate (MMA), glycolmethacrylate (GMA) or an epon-like plastic. The preferred method used for metabolic bone disease research and diagnosis is MMA. Plastic is not a common embedding medium in clinical laboratories but is useful in research settings. It is also possible to produce frozen sections from an undecalcified bone biopsy. There are silver stains which demonstrate bone and osteoid in a decalcified, paraffin wax-embedded bone section (Tripp & McKay, 1972) but many researchers choose the MMA-embedded section.

It is not practical to bisect an iliac crest trephine biopsy if both paraffin wax and plastic embedding methods are employed in the laboratory. Metabolic bone disease laboratories usually prefer a whole trephine bone core for plastic embedding. Needle biopsies should remain whole for paraffin wax or plastic methods.

Amputation specimens

Large amputation specimens are usually taken as a result of tumor, chronic osteomyelitis or gangrene. These specimens are usually delivered to the laboratory immediately after removal. They are often not in a sealed container, without fixative and must be dealt with as soon as possible, either in the mortuary or laboratory. The majority of the limb is usually discarded or saved/fixed (if requested by the patient) and the area or lesion with actual or suspected involvement in the disease process is retained for final evaluation. Skin, excess muscle and connective tissue should be cut away from the lesion if possible. Excess bone or a joint disarticulation above and below the lesion should be performed so that fixation is adequate. The relevant portions should be immersed into a large volume of fixative ensuring complete fixation. If it is not possible to inspect the specimen for several hours after receipt, it should be refrigerated at 4°C or placed in fixative as a whole and kept at 4°C. Placing it in fixative prior to trimming helps in managing the trimming and prevents autolysis of the outer layers of the specimen. The

mortuary has a dual advantage for both limb storage and subsequent sample preparation on an available autopsy table. Whenever possible, specimen radiography of large bone specimens helps select the lesion/diseased area for trimming to a smaller sample size for processing.

Resection/replacement specimens

Benign or low grade malignant tumors and arthritic femoral heads resemble large biopsy specimens and frequently have an established diagnosis so are usually considered less urgent. In femoral head or knee replacement surgeries, the bone specimen removed from the patient is usually received in the laboratory whole. Either prior to or after partial fixation, a wedge-shaped sample is cut from the whole specimen using a Stryker bone saw or a heavy-duty X-ACTO knife. This wedge shaped sample is placed back into fixative for 24–48 hours and then decalcified, processed and sectioned for pathologic evaluation.

Fixation

Unless immediate diagnosis is needed using cryomicrotomy, all bone specimens must be completely fixed before subjecting them to any decalcification and processing procedures. Complete fixation helps protect the bone and surrounding soft tissue from the damaging effects of acid decalcification. Ten percent neutral buffered formalin (NBF) is suitable for both paraffin and non-tetracycline labeled bone. It should be noted that fixation proceeds faster by reducing the size of the bone, opening the bone, and removing excess skin and soft tissue surrounding the lesion. Large specimens can be bisected or reduced in size by sawing into multiple slabs and immersing into fixative immediately, or no longer than 48 hours after initial fixation. Once cut into smaller pieces, the samples should be placed into fresh fixative.

For MMA embedding, 10% NBF is generally used for fixation. Alcoholic formalin or 70% ethanol fixation is the fixative of choice for tetracycline labeled bone. Alcohol based fixatives are not recommended for bone destined for acid decalcification as alcohol can slow or prevent decalcification. Fixatives containing chloroform (Carnoy's) and mercury (B5, Zenker's, Susa's), including substitutes for them, should be avoided in specimens to be radiographed since these fixatives tend to make bone radio-opaque and unsuitable for specimen interpretation.

Sawing

Good saws are essential pieces of equipment in a bone histology laboratory. Other than surgical saws, there are a range of hobby shop or handyman's bench saws which are designed to cut through stones, plastic and some thin metals. These saws cut through cortical bone slowly with cuts no deeper than 7.5 cm. Dry saws may need slight modifications to prevent blade slippage when cutting wet, fatty bone. Water-cooled saws prevent heat damage to bone due to high speed sawing, and are capable of full length cuts through long bones and appendages, e.g. femur and tibia. Buehler Isomet Low Speed Saws (Buehler Ltd, USA) are used for trimming specimens and cutting bones embedded in plastics. This type of saw has a thin diamond-impregnated blade with a water cooling bath and is ideal for bisecting biopsies (when required), and larger bones, depending on the bone specimen diameter. It can make precise, debris-free cuts through 8 mm thick bone cores, cortical or trabecular bone and MMA-embedded bone specimens. Scalpel blades, fretted wire or jewelers' saws have been used to cut biopsies with damaging results. These cutting devices can crush or fracture fine trabeculae, creating 'fracture artifact' and force bone fragments into marrow spaces, damaging the bone histology.

Suitable blade specifications on small saws are 0.5 cm width and 12 to 16 teeth per inch (tpi), making finer, cleaner cuts than a larger saw blade 1.25 cm wide with 6 tpi. Blades in specifications needed are available from tool companies.

Soft tissues and dense connective tissue, e.g. tendons, should be removed before sawing or the sample will drag through the blade. The first cut is made through the mid-plane, then approximately 3–5 mm thick slabs are cut parallel to the first cut. A saw guide plate or wooden block held against

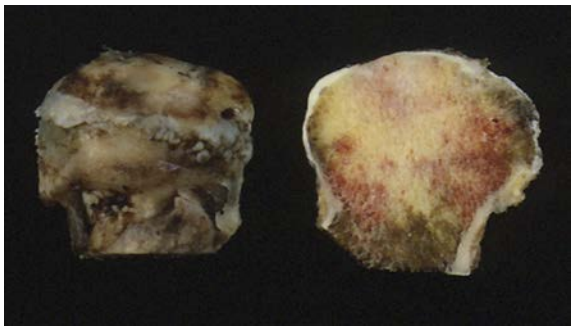


Fig. 17.4 Incomplete fixation. Bone showing some fixation, tan color, with the red/pink areas showing incomplete fixation.

the first cut edge ensures an even slice. It is safer for workers to hold thinner bones between two wooden blocks which will not ruin blades. Sawing should be at a slow even rate to match the speed of blade cutting into the bone. Pushing the bone produces uneven cuts, and may jam or break a blade.

Bone slabs should be fixed for an additional 24–48 hours, especially if they appear pinkish-red or partially fixed. After sawing, any bone dust or debris adhering to slab surfaces can be cleaned away using a slow stream of water and a wet paper towel to brush off the debris. Care must be taken not to push debris into marrow spaces or to wash slabs excessively before the bone is totally fixed (Fig. 17.4).

Fine-detail specimen radiography

Radiographs of bone slabs, blocks or fragments are useful for four main purposes:

1. To examine the nature and extent of a lesion.
2. To provide a diagram of a lesion prior to block selection for processing.
3. To check progress of decalcification, i.e. decalcification endpoint test.
4. To confirm the presence of foreign materials, e.g. prosthetic devices, metal or glass fragments implanted by trauma.

Thin bone slices give sharper image radiographs than a whole specimen or clinical radiographs (Fornasier, 1975).

Many non-specialist departments of pathology use their in-house radiology department. X-ray (digital) images may also be acquired from other areas of the facility, e.g. surgery. It is common practice in cancer or orthopedic hospitals to have a portable, fully-enclosed X-ray system in surgery to ensure full removal of tumor or diseased areas. One example of this type of unit is the Faxitron (Faxitron Inc., USA) cabinet X-ray system which can be safely used for specimen X-rays of bone in clinical and research settings. A special door interlock safety device automatically turns off the X-ray beam if the door is opened during operation. This instrument produces digital and real time images. When using a Faxitron for decalcification checks, a bone slab should be first radiographed using the automatic exposure timer and then exposure time, kV and mA are recorded. This eliminates guesswork for a first exposure and provides the correct exposure time and kV for a repeat radiograph or for subsequent manual exposures of adjacent bone slabs of the same thickness (Fig. 17.5).

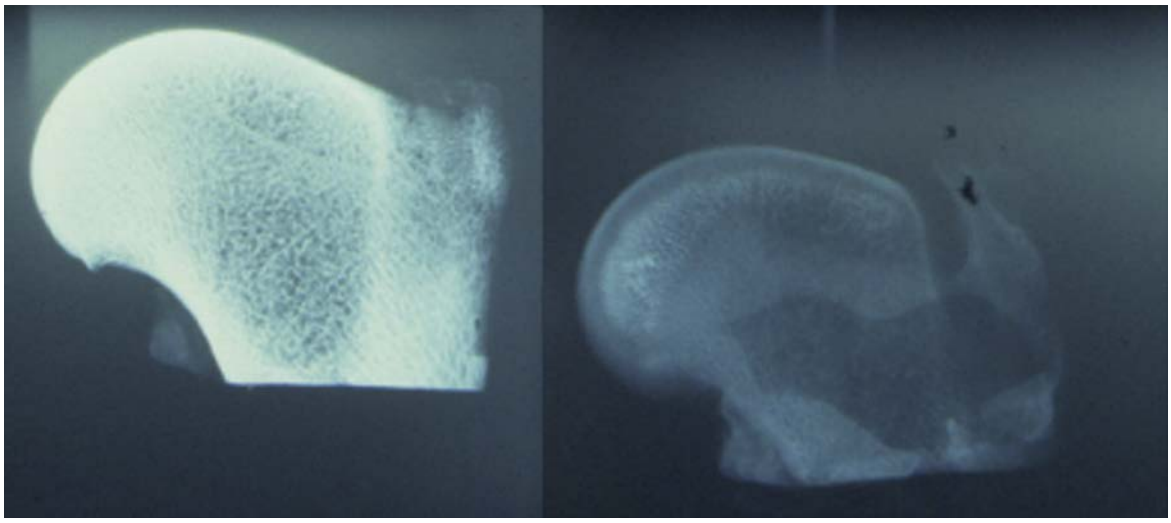
Soft tissues, cartilage and tumors are more easily seen in underexposed radiographs which are useful for evaluating surrounding soft tissue involvement by a bone tumor, e.g. osteosarcoma.

Area selection for embedding

In urgent cases of suspected tumor or infection, an attempt should be made to select a sample with the least mineralization in order to provide the quickest possible diagnosis. These pieces can be fixed, rapidly decalcified and processed to meet urgent clinical requirements.

The ideal thickness of larger bone pieces is 3–5 mm. If bone slabs are too thick, both decalcification and processing are prolonged whilst overly thin bone slabs, less than 2 mm, tend to become brittle and bend during processing and embedding which may cause the tissue to pop out of the paraffin wax block during sectioning. The dense collagen matrix tends to prevent adequate paraffin wax penetration and thin pieces are not held firmly in the softer paraffin wax embedding media during microtomy.

As mentioned earlier a radiographic diagram or map can be used to locate specific areas of interest of the lesion for processing.



a b

Fig. 17.5 An example of a Faxitron X-ray (**a**) before decalcification and (**b**) after decalcification is completed. The bright white in the sample is calcium: as the calcium is removed, the bone becomes more translucent.

Decalcification

In order to obtain satisfactory paraffin sections of bone, inorganic calcium must be removed from the organic collagen matrix, calcified cartilage and surrounding tissues. This decalcification process is carried out by chemical agents; either acids to form soluble calcium salts, or chelating agents which bind to calcium ions. Even after decalcification, the dense collagen of cortical bone is remarkably tough and tends to harden more after paraffin processing. Occasionally, small foci of calcifications in paraffin wax-embedded or frozen tissues can be sectioned without noticeable damage to the knife or disruption of surrounding tissue. After hematoxylin staining, these foci usually appear cracked and as dark purple granular masses with lighter purple halos.

The choice of decalcifier is influenced by four inter-dependent factors:

- Urgency of the case.
- Degree of mineralization of the bone.
- Extent of the investigation required.
- Staining techniques required.

Any acid, however well buffered, has some damaging effects on tissue stain avidity. This problem increases with the acidity of solutions, i.e. lower pH and length of decalcification period. Consequently, rapid decalcifiers are more likely to adversely affect any subsequent staining, especially if not fixed completely. This is most noticeable in cell nuclei with the failure of nuclear chromatin to take up hematoxylin and other basic dyes as readily as soft tissues never exposed to acid solutions. The staining using acid dyes is also less affected, but eosin (an acid dye) can stain tissue a deep, unpleasant, brick red without the preferred three differential shades. These effects on H&E staining can be reduced by performing the decalcification endpoint test, post-decalcification acid removal and adjustment of the stain procedure.

Decalcifying agents

As noted previously there are two types of decalcifiers, acids and chelating agents, although Gray (1954) lists over 50 different mixtures. Many of these mixtures were developed for specific purposes with one used as a fixing and dehydrating agent. Other mixtures contain reagents, e.g. buffer salts, chromic acid, formalin or ethanol, intended to counteract the undesirable swelling effects which acids have on tissues. Many popular mixtures used today are from the original formulas developed many years ago (Evans & Krajian, 1930;

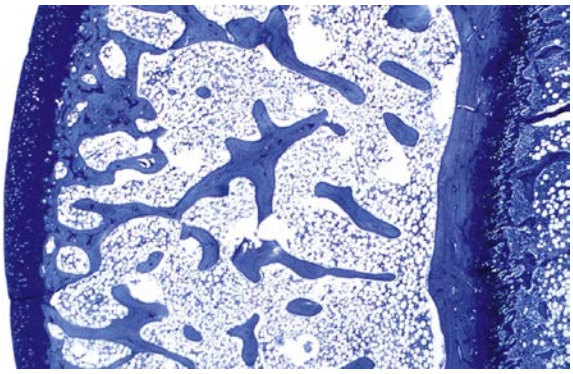


Fig. 17.6 Toluidine blue-stained articular cartilage and bone. Formic acid-decalcified rat femur in a paraffin wax section.

Kristensen, 1948; Clayden, 1952). For most practical purposes, laboratories prefer simpler and premixed solutions for routine work. Provided the bone is totally fixed and treated with a decalcifier suitable for removal of the amount of mineral present, the simple mixtures work as well or better than more complex mixtures.

Acid decalcifiers

Acid decalcifiers can be divided into two groups: strong (inorganic) and weak (organic) acids. As Brain (1966) suggested, many laboratories keep an acid from each group available for either rapid diagnostic or slower, routine work (Fig. 17.6).

Strong inorganic acids, e.g. nitric, hydrochloric

These may be used as simple aqueous solutions with recommended concentrations of 5–10%. They decalcify rapidly, cause tissue swelling and can seriously damage tissue stainability if used for longer than 24–48 hours. Old nitric acid is particularly damaging and should be replaced with fresh stock. Strong acids, however, tend to be more damaging to tissue antigens for immunohistochemical staining and enzymes may be totally lost.

Strong acids are used for needle and small biopsy specimens allowing rapid diagnosis within 24 hours or less. They can be used for large or heavily mineralized cortical bone specimens with decalcification progress carefully monitored by a decalcification endpoint test (Callis & Sterchi, 1998). The following is a list of strong acid decalcifying solutions; the formulas and preparations are available in Bancroft & Gamble (2008).

1. Aqueous nitric acid, 5–10% (Clayden, 1952).
2. Perenyi's fluid (Perenyi, 1882).
3. Formalin-nitric acid (use inside a fume hood).

Weak organic acids, e.g. formic, acetic, picric

Of these, formic is the only weak acid used extensively as a primary decalcifier. Acetic and picric acids cause tissue swelling and are not used alone as decalcifiers but are found as components in Carnoy's, Bouin's and Zenker's fixatives. These fixatives will act as incidental, although weak, decalcifiers and could be used in urgent cases with only minimal calcification. Formic acid solutions can be aqueous (5–10%), buffered or combined with formalin. The formalin-10% formic acid mixture simultaneously fixes and decalcifies, and is recommended for small bone pieces or needle biopsies. However, it is still advisable to have complete fixation before any acid decalcifier is used. The salts, sodium formate (Kristensen, 1948) or sodium citrate (Evans & Krajian, 1930), are added to formic acid solutions making 'acidic' buffers. Buffering is used to counteract the injurious effects of the acid. However, in addition to low, 4–5% formic acid concentration, increased time is needed for complete decalcification. Formic acid is gentler and slower than HCl or nitric acids, and is suitable for most routine surgical specimens, particularly when immunohistochemical staining is needed. Formic acid can still damage tissue, antigens and enzyme staining, and should be endpoint tested. Decalcification is usually complete in 1–10 days depending on the size, type of bone and acid concentration. Dense cortical or large bones have been effectively decalcified with 15% aqueous formic acid and a 4% hydrochloric acid–4% formic acid mixture (Callis & Sterchi, 1998). The following is a list of weak acid decalcifying solutions; the formulas and preparations are available in Bancroft & Gamble (2008).

1. Aqueous formic acid.
2. Formic acid-formalin (after Gooding & Stewart, 1932).
3. Buffered formic acid (Evans & Krajian, 1930).

Chelating agents

The chelating agent generally used for decalcification is ethylenediaminetetraacetic acid (EDTA). Although EDTA is nominally 'acidic', it does not act like an inorganic or organic acid but binds metallic ions, notably calcium and magnesium. EDTA will not bind to calcium below pH 3 and is faster at pH 7–7.4; even though pH 8 and above gives optimal binding, the higher pH may damage alkali-sensitive protein linkages (Callis & Sterchi, 1998). It binds to ionized calcium on the outside of the apatite crystal and as this layer becomes depleted more calcium ions reform from within; the crystal becomes progressively smaller during decalcification. This is a slow process which does not damage tissues or their stainability. When time permits, EDTA is an excellent bone decalcifier for enzyme staining and electron microscopy. Enzymes require specific pH conditions in order to maintain activity, and EDTA solutions can be adjusted to a specific pH for their staining. EDTA does inactivate alkaline phosphatase, but activity can be restored by the addition of magnesium chloride.

EDTA, EDTA disodium salt (10%) or EDTA tetrasodium salt (14%) are approaching saturation and can be simple aqueous or buffered solutions at a neutral pH of 7–7.4, or added to formalin. EDTA tetrasodium solution is alkaline and the pH should be adjusted to 7.4 using concentrated acetic acid. The time required to totally decalcify dense cortical bone may be 6–8 weeks or longer, although small bone spicules may be decalcified in less than a week. The formulas and preparations are available in Bancroft & Gamble (2008).

1. Formalin-EDTA (Hillemann & Lee, 1953).
2. EDTA (aqueous), pH 7.0–7.4.

Proprietary decalcifiers

The components in proprietary decalcifying solutions are often trade secrets. Manufacturers provide Material Safety Data Sheets (MSDS) which frequently indicate the type and concentration of acid. These usually indicate if a solution is rapid or slow and give decalcification instructions and warnings against prolonged use. Rapid proprietary solutions usually contain hydrochloric acid (HCl), whereas slow proprietary mixtures contain buffered formic acid or formalin/formic acid. A study (Callis & Sterchi, 1998) found that dilution of a proprietary HCl solution was not deleterious for effective decalcification or staining, and this is an option if a strong mixture is considered too concentrated. Chelating reagents such as EDTA mixtures are also available pre-mixed. Although proprietary mixtures have no obvious advantages over solutions prepared in laboratories, their use is more common in busy laboratories because they are reliable, save time and expense, and address safety issues by eliminating handling and storage of concentrated acids.

Factors influencing the rate of decalcification

Several factors influence the rate of decalcification and there are ways to speed up or slow down this process. The concentration and volume of the active reagent, including the temperature at which the reaction takes place, are always important. Other factors which contribute to how fast bone decalcifies are the age of the patient, type of bone, size of specimen and solution agitation. Mature cortical bone decalcifies slower than immature, developing cortical or trabecular bone. Another factor of mature bone is that the marrow may contain more adipose cells than a young bone. This requires diligent attention to make sure specimens stay immersed in decalcification solution. Of all the above factors, the effectiveness of agitation is open to debate.

Concentration of decalcifying agent

Generally, more concentrated acid solutions decalcify bone more rapidly, but are more harmful to the tissue. This is particularly true of aqueous acid solutions as various additives, e.g. alcohol or buffers which protect tissues, may slow down the decalcification rate. Remembering that 1 N and 1 M solutions of HCl, nitric or formic acid are equivalent, Brain (1966) found that 4 M formic acid decalcified twice as fast as a 1 M solution without harming tissue staining, and felt it was advantageous to use the concentrated formic acid mixture. With combination fixative-acid decalcifying solutions, the decalcification rate cannot exceed the fixation

rate or the acid will damage or macerate the tissue before fixation is complete. Consequently, decalcifying mixtures should be a compromise which balance the desirable effects, e.g. speed, with the undesirable effects, e.g. maceration and impaired staining.

In all cases, total depletion of an acid or chelator by their reaction with calcium must be avoided. This is accomplished by using a large volume of fluid compared with the volume of tissue (20:1 is recommended), and by changing the fluid several times during the decalcification process. Brain (1966) pointed out that if a sufficiently large volume of fluid is used (100 ml per g of tissue) it is not necessary to renew the decalcifying agent because depletion is less likely in a larger volume. Small numbers and similar sized specimens in one container are preferred.

Ideally, acid solutions should be endpoint tested and changed daily to ensure that the decalcifying agent is renewed and tissues are not left in too long, or overexposed to acids i.e. 'over-decalcified'.

Temperature

Increasing the temperature accelerates many chemical reactions, including decalcification, but it also increases the damaging effects which acids have on tissue. At 60°C, the bone, soft tissues and cells may become completely macerated almost as soon as they are decalcified.

The optimal temperature for acid decalcification has not been determined, although Smith (1962) suggested 25°C as the standard temperature, but in practice a room temperature (RT) range of 18–25°C is acceptable. Conversely, lower temperature decreases reaction rates and Wallington (1972) suggested that tissues not completely decalcified at the end of a working week could be left in acid at 4°C over a weekend. This practice may result in 'over-decalcification' of tissues, even with formic acid. A recommendation is to interrupt decalcification by briefly rinsing acid off the bone, immersing it in NBF, removing it from and rinsing off the fixative, resuming decalcification on the next working day. Microwave, sonication and electrolytic methods produce heat and must be carefully monitored to prevent excessive temperatures which damage tissue (Callis & Sterchi, 1998).

Increasing the temperature also accelerates EDTA decalcification without the risk of maceration. However, it may not be acceptable for preservation of heat-sensitive antigens, enzymes or electron microscopy work. Brain (1966) saw no objection to decalcifying with EDTA at 60°C if the bone was well fixed.

Agitation

The effect of agitation on decalcification remains controversial even though it is generally accepted that mechanical agitation influences fluid exchange within, as well as around tissues with other reagents. Therefore, it would be a logical assumption that agitation speeds up decalcification and studies have attempted to confirm this theory. Russell (1963) used a tissue processor motor rotating at one revolution per minute and reported the decalcification period was reduced from 5 days to 1 day. Others, including Clayden (1952), Brain (1966), and Drury & Wallington (1980) repeated or performed similar experiments and failed to find any time reduction. The sonication method vigorously agitates both specimen and fluid, and one study noted cellular debris found on the base of a container after sonication could possibly be important tissue shaken from the specimen (Callis & Sterchi, 1998). Gentle fluid agitation is achieved by low speed rotation, rocking, stirring or bubbling air into the solution. Even though findings from various studies are unresolved, agitation is a matter of preference and not harmful as long as tissue components remain intact.

Suspension

The decalcifying fluid should be able to make contact with all surfaces of a specimen and flat bone slabs should not touch each other or the bottom of a container. Bone samples can be separated and suspended in the fluid with a thread, placed inside cloth bags tied with thread or preferably in a cassette. The cassette will provide identification without having to prepare tags for bag suspension. Some workers have cleverly devised perforated

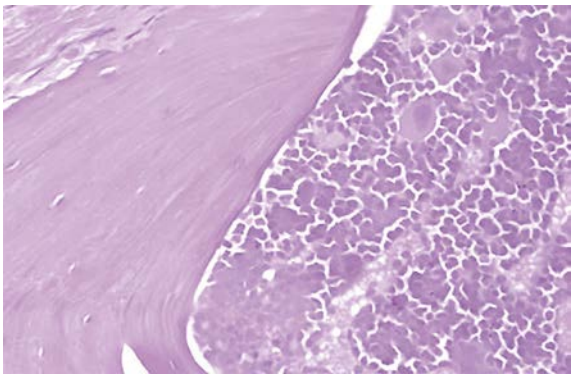


Fig. 17.7 Over-decalcified tissue demonstrating the lack of nuclear staining and only one shade of eosin.

plastic platforms which raise samples above the container bottom allowing fluid access to all of the bone surfaces.

Completion of decalcification

Ideally, bone should be taken from the delcalcifying solution as soon as all the calcium has been removed from it. Although the outer parts of a sample will possibly be overexposed to the acid, these usually stain no differently from the inner portions which are the last to be decalcified. Tissues decalcified in acids for long periods, or in high acid concentrations are more likely to show the effects of over-decalcification whether or not all the mineral has been removed (Fig. 17.7).

Consequently, it is important for a laboratory to control a decalcification procedure by using a decalcification endpoint test to know when calcium removal is complete and, if incomplete, renew the decalcifying agent. It is recommended that all laboratories perform endpoint testing. When using formic, HCl or nitric acids, daily testing is recommended unless near the endpoint, then test every 3–5 hours when possible. With EDTA, weekly tests are sufficient unless solution changes are more frequent. It is recommended that EDTA be changed frequently initially as the reaction with calcium starts quicker and then slows down as the calcium in the tissue is depleted. Minimally calcified tissues and needle biopsies decalcified by a strong acid may only need one test. It is wise practice for a laboratory which performs a high number of bone biopsy specimens to establish a decalcification time range which depends on the amount of cortical bone present. Once the process is established it would eliminate guessing, testing and handling of these delicate samples. Biopsy decalcification is fairly consistent on multiple-sized biopsies within an hour or two of acid exposure. Wrapping a bone needle biopsy in tissue paper and leaving it wrapped until embedding prevents any loss of cells or tissue during the washing or decalcification process. Sponges sometimes pull cells from a sample when washing and decalcifying. Often these may be urgent cases where a shorter decalcification time is allowed, but the sample must be carefully treated and incomplete decalcification is still possible. If tissue is still slightly under-decalcified after paraffin wax embedding and sectioning, surface decalcification can be done. Problem blocks should be identified so that proper treatment is given should further microtomy be requested.

Decalcification endpoint test

There are several methods for testing the completion of decalcification, two are considered to be the most reliable: specimen radiography using an X-ray unit and the chemical method to test acids and EDTA solutions. Another method first used to test nitric acid is a weight loss, weight gain procedure which provides relatively good, quick results with all acids and EDTA (Mawhinney et al., 1984; Sanderson et al., 1995). Although still used, 'physical' tests are considered inaccurate and damaging to tissues. Probing, 'needling', slicing, bending or squeezing tissue can create artifacts, e.g. needle tracks, disrupt soft tumor from bone or cause false-positive microfractures of fine trabeculae, a potential misdiagnosis.

Methods for chemical testing of acid decalcifying fluids detect the presence of calcium released from bone. When no calcium is found or the result is negative, decalcification is said to be complete and may entail using one extra change of decalcifier after actual completion. EDTA can be chemically endpoint tested by acidifying the used solution, this forces EDTA to release calcium for precipitation by ammonium oxalate (Rosen, 1981).

Calcium oxalate test (Clayden, 1952)

This method involves the detection of calcium in acid solutions by precipitation of insoluble calcium hydroxide or calcium oxalate. It is unsuitable for solutions containing over 10% acid even though these could be diluted and result in a less sensitive test.

Solutions

Concentrated amonium hydroxide.
Saturated aqueous ammonium oxalate.

Method

1. Take 5 ml of used decalcifying fluid, add a piece of litmus paper or use a pH meter with a magnetic stirrer.
2. Add ammonium hydroxide drop by drop, shaking after each drop, until litmus or pH meter indicates solution is neutral (pH 7).
3. Add 5 ml of saturated ammonium oxalate and shake well.
4. Allow solution to stand for 30 minutes.

Result

If a white precipitate of calcium hydroxide forms immediately after adding the ammonium hydroxide, a large quantity of calcium is present, making it unnecessary to proceed further to step 3, which would also be positive. Testing can be stopped and a change to fresh decalcifying solution made at this point. If step 2 is negative or clear after adding ammonium hydroxide, then proceed to step 3. If precipitation occurs after adding the ammonium oxalate, less calcium is present. When a smaller amount of calcium is present, it takes longer to form a precipitate in the fluid; if the fluid remains clear after 30 minutes, it is safe to assume decalcification is complete (Fig. 17.8).

Fig. 17.8 Example of endpoint testing with the calcium oxalate test, demonstrating complete decalcification (clear tube) and incomplete decalcification (cloudy tube).

Radiography is the most sensitive test for detecting calcium in bone or tissue calcification and is described earlier in this chapter under 'Fine-detail specimen radiography'.

Treatment following decalcification

Acids can be removed from tissues or neutralized chemically after decalcification is complete. Chemical neutralization is accomplished by immersing decalcified bone into either saturated lithium carbonate or 5–10% aqueous sodium bicarbonate solution for several hours. Many laboratories simply rinse the specimens with running tap water for a period of time. Culling (1974) recommended washing in two changes of 70% alcohol for 12–18 hours before continuing with dehydration in processing; this avoids contamination of dehydration solvents even though the dehydration process would remove the acid along with the water.

Adequate water rinsing can generally be done in 10 minutes for small samples and larger bones need 20–40 minutes to prevent a delay in processing. Samples needing immediate processing, e.g. needle biopsies, can be blotted or quickly rinsed to remove acid from surfaces before proceeding to the first dehydrating fluid. It is important to avoid contaminating the first dehydrating fluid with acids, and washing bones even for a short time is good practice particularly with large bone slabs.

Tissues decalcified in EDTA solutions should not be placed directly into 70% alcohol as this causes residual EDTA to precipitate in the alcohol and within the tissue. The precipitate does not appear to affect tissue staining since EDTA is washed out during these procedures, but may be noticeable during microtomy or storage when a crystalline crust forms on the block surface. A water rinse after decalcification or overnight storage in formal saline, NBF or PBS should prevent this.

Processing decalcified bone

In today's laboratories, automated computerized processors with vacuum and pressure options have improved the efficiency and quality of tissue processing, particularly bone. Solvents used for dehydration (ethanol, isopropanol, reagent and proprietary alcohol mixtures) and clearing (xylene, xylene substitutes) work well for bone and soft tissue processing. Paraffin waxes developed in recent years have been improved by the addition of plastic polymers and other chemicals which allow better wax penetration and sectioning. Decalcified bone sectioning is made easier after infiltration and embedding in a harder paraffin wax which will give firmer support of the bone during sectioning. Small bone and needle biopsies containing little cortical bone can be processed with soft tissues.

Oversized, thick bone slabs require an extended processing schedule in order to obtain adequate dehydration, clearing and paraffin wax infiltration. Some laboratories specialized in orthopedic work find it advantageous to dedicate one processor to extended processing schedules for bone which then does not interfere with routine daily soft tissue processing. With an enclosed automatic processor, time in each dehydrating solution, clearing solvent and paraffin wax may vary from 2 to 4 hours depending on the size if the bone sample. Modern embedding methods using metal molds with plastic tissue cassettes have all but eliminated the necessity to mount the paraffin wax-embedded tissues on wood, hard rubber blocks or metal chucks. A labeled cassette contains the tissue throughout processing and, after embedding, the plastic back of a block fits into a microtome cassette clamp. Macro-cassette systems including larger cassettes, molds and a special block holder are available for sledge microtomes. Specimen size is the limiting factor for embedding with cassettes, and with a little creativeness the oversized bone can be embedded in a paraffin wax filled metal pan or similar container, a warm hard wooden block is then placed directly on top of the bone and it is all allowed to harden in place. This results in the wood being embedded directly in the block, which can then be used to clamp the specimen tightly in the microtome, avoiding holding onto softer paraffin wax, which can crack under excessive clamping pressure.

Microtomy of bone

Microtomes and knives

Bone biopsies and smaller primarily cancellous bone blocks can be cut on any properly maintained microtome. Many newer microtomes are more powerful, heavier and automated, making them capable of sectioning both paraffin wax and plastic bone blocks. Oversized and exceptionally hard, dense bone samples, too difficult to cut on a smaller microtome, are easier to section on a large sledge or heavy duty motorized sliding microtome (Polycut, Leica) or a laser cutting microtome (Rowiak GmbH).

There is a wide choice of good microtome blades including heavy 'c' profile steel and the popular disposable blades. The disposable knives are convenient, extremely sharp, single-use blades capable of sectioning properly decalcified and processed paraffin wax-embedded bones. Newer microtomes come equipped with disposable blade holders, or disposable blade holder inserts can be purchased for older model microtomes. High-profile disposable blades are slightly thicker and wider than the low-profile blades and tend to 'chatter' or vibrate less when cutting denser bones. Heavier steel knives range in length from 16 to 18 cm for small microtomes and from 200 to 300 cm for base sledge microtomes with specially designed blades for the Polycut. Steel knives need frequent sharpening and an automatic knife sharpener is a cost-effective, time-saving device when these are used routinely. An automatic knife sharpener is a rare find but a multiple plate design can also sharpen tungsten carbide knives which are used for undecalcified bone cryotomy and plastic-embedded tissues. Sharpening a tungsten carbide knife frequently can be expensive and a knife sharpener can save

time and money. Unlike steel knives, tungsten carbide knives need to be reconditioned after multiple sharpenings.

Microtome sectioning of bone

Small bone samples and biopsies usually section well with knife angles set for routine soft tissue microtomy. Generally, disposable blades work best at the manufacturer's recommended angle settings for their high or low profile blades. Slight adjustment of a knife angle can be attempted if dense cortical bone sectioning is not working with the routine soft tissue knife angle, it can be increased or decreased at a microtomist's discretion. Knives must be changed frequently, sometimes after cutting one ribbon or a few sections of cortical bone. When sectioning any bone sample, a sharp knife is necessary in order to get flat, uncompressed, wrinkle free sections, as well as the patience and good microtomy skills of the operator.

Longitudinal sections of cortical bone may section better when the knife cuts along the length of the bone oriented at right angles to the knife. A rectangular shaped piece of bone can be embedded or oriented in a block holder so that a smaller corner of sample is cut first with the wider area cut last. This helps reduce knife vibration and potential gouging of bone out of the paraffin wax block. When cartilage is present, it should be located near the top of a block or angled in a way to avoid compression of the softer cartilage and paraffin wax into the denser bone, creating wrinkles. Generally, hard tissues cut easier if cooled by a melting ice block allowing water penetration into the tissue surface. Extensive soaking causes visible tissue swelling away from the block face, and even though the tissue cuts more easily, the sections fall apart on the water bath. A flat ice block made with water-filled polyethylene storage bags keeps blocks dry during cooling, or paraffin wax bone blocks can be cooled in a −20°C freezer for a short time. Larger blocks can be cut at room temperature as long as the room is not overly warm or humid. If using a tape method to obtain a difficult section, the blocks must be at room temperature and dry so that the tape adheres to the block.

The optimal thickness for bone sections is the same as that for soft tissues; 4–5 µm is cut routinely from adequately processed blocks. Bone marrow biopsies should be cut at 2–3 µm for hematopoietic cell identification, and sliding microtome sections may vary from approximately 5–8 µm.

Flattening and adhesion

Bone sections adhere to slides well when glass surfaces are coated with adhesive. Slides come in all types of coating with different levels of tissue adhering properties. All work well but a strong charge or coating is preferred when working with bone. Some caution needs to be taken when using strongly charged slides. Strongly charged slides grab the tissue section so tight that it is difficult to manipulate the section to remove excess water and folds. If only plain, uncoated slides are available the following is a simple coating method: wash slides in soap and water, rinse soap off completely, dip in a gelatin and potassium dichromate 'subbing' solution, air dry, and store in a clean dry box until needed (Drury & Wallington, 1980). When sectioning numerous bone blocks, 10 ml of the chrome subbing solution can be added to a two liter water bath or simply add a few gelatin granules to the water as it is heating. If sections are persistently non-adherent, a solution containing amylopectin, a starch (Steedman, 1960), or a high molecular weight 225 bloom gelatin in the chrome subbing mixture may be more successful. Gelatin should be used sparingly or an excess coating is stained by hematoxylin giving an unsightly blue background underneath and around the sections.

Whilst floating on water, cartilage and bone sections can expand more than the paraffin wax or other tissue components, and small folds may form as the sections dry. When this occurs, the water bath temperature should be lowered to 10–15°C below the paraffin wax melting point. Flattening a section by mounting it in excess water on the slide, then holding the slide against a hot plate to melt the wax and evaporate the water must be used with caution. Bone sections may 'explode' apart, displacing

cortical bone from trabecular bone and ruining the gross morphology. Reducing the surface tension of water by floating a section on room temperature 10% ethanol, picking up the section on a slide, then immediately but slowly lowering the section into a warm water bath allows a section to flatten gently. If cartilage curling is a problem, drying sections flat at 37°C overnight or longer may solve this problem. Most bone sections flatten and dry without problems or special treatment provided the tissue has been properly processed and sectioned with a sharp knife.

Frozen sections of bone

There are times when a frozen section is needed. This can be done without decalcification on small bone specimens. It is more difficult to produce quality sections of large undecalcified specimens and these are usually not ideal for image analysis but adequate for diagnosis. A small undecalcified bone specimen slide can be achieved by using a device called CryoJane™ (Leica). It uses specially coated slides, tape and light activation. This device helps hold the tissue together and reduces chatter on frozen sections of difficult tissues.

When able to decalcify the bone for cryotomy acid-decalcified tissues must be thoroughly washed in water, stored in formal saline containing minimal amounts of sucrose (3–5%) or PBS with 3–5% sucrose, at 4°C before freezing. This helps avoid any residual acid in the tissue from corroding the metal knife. However, higher percentages of sucrose may prevent the tissue from fully freezing or freezing unevenly, causing soft spots in the tissue which will create thick/thin appearing sections, or the tissue will fall out due to improper freezing. Frozen sections from trephine and needle biopsies of cortical and trabecular bone can be cut with ease and minimal section damage using a modern cryostat, patience, a slow steady cutting speed and a tungsten carbide-tipped steel knife. A knife with a tungsten carbide edge is much harder than a steel one and cuts calcified bone without fragmenting the section or damage to the knife edge.

For demonstration of bone marrow cells, tumor and calcified bone components, the hematoxylin stains cell nuclei and mineralized bone intensely blue, and eosin stains osteoid and other soft tissues shades of red. Some bone with metabolic diseases (e.g. Paget's, renal osteodystrophy and hyperparathyroidism), showing advanced changes, or diseased bone with moderate to severe osteomalacia, can be rapidly diagnosed on an H&E-stained frozen section. Other stains which can be used with frozen bone sections, include a modified Romanowsky method for patterns in bone remodeling and cartilage development (Dodds & Gowen, 1994), enzyme and immunohistochemical methods. Unstained sections can be examined with polarized light to see woven and lamellar patterns in bone.

Laboratories not using plastic embedding techniques may find bone cryotomy a valuable addition to their facility. Frozen sections permit rapid diagnosis of some bone diseases. Rapid or 'snap' freezing bone samples in liquid nitrogen-cooled isopentane (2-methylbutane), must be performed carefully as some bones can shatter in the extremely cold (–120°C) temperature. Bone coated with 4% aqueous polyvinyl alcohol (PVA, water soluble, 124,000 MW) or embedded in optimum cutting temperature compound (OCT) can be snap frozen in a dry ice/isopentane bath (–70°C) gently and without shattering. Hexane can be substituted for isopentane (Dodds & Gowen, 1994).

A suitable technique is:

1. Mount bone on cork or embed in a cryomold with OCT.
2. Snap freeze carefully in 'syrupy' (thawing) isopentane cooled by liquid nitrogen (–120°C) or with dry ice/isopentane (–70°C).
3. Place bone in cryostat at –30 to –35°C. Remount frozen bone onto a metal chuck with OCT to provide maximum stability during sectioning.
4. Cut section at 5–7 µm, pick up the section on a slide and fix with fixative of choice. Post-fixation in 95% alcohol for 5 minutes removes any fat.
5. Stain in Harris, Gill II or Gill III hematoxylin for 1 minute or longer for desired intensity. Rinse with water or a blueing reagent to 'blue' section; avoid ammonia water.
7. Stain in 1% alcoholic eosin for approximately 10–30 seconds or desired intensity.
8. Dehydrate, clear and mount in permanent mounting medium.

Notes

a. Formalin-fixed biopsies can be rinsed, immersed in 5–10% sucrose for 1–8 hours at 4°C to replace water before freezing and improve sectioning (cryoprotection). Higher concentrations of sucrose for cryoprotection may delay freezing and cause uneven freezing. Optimization and practice should be used to determine the amount of sucrose needed.

b. Fresh frozen sections can be fixed, rinsed and then decalcified in 10% EDTA before immunostaining.

c. Enzyme staining can be done on fixed or unfixed sections.

d. Any hematoxylin can be used with staining intensity optimized for worker preference.

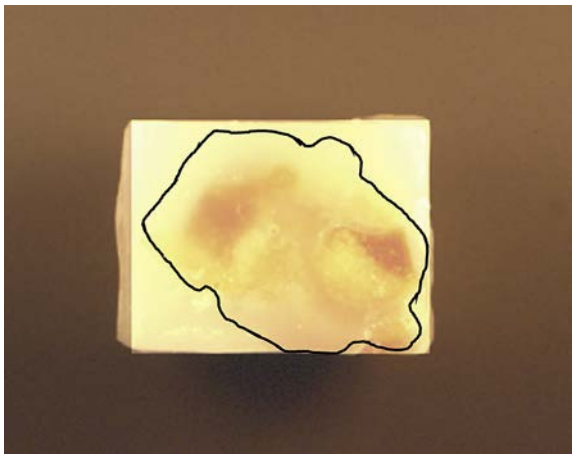


Fig. 17.9 Block with improper fixation, decalcification, and processing.

Troubleshooting

Inadequate fixation, decalcification and processing can create multiple microtomy problems which may not be recognized until microtomy. Blocks may be received from other facilities where decalcification endpoint checks were not done or the processor solutions were old. There are certain exercises which can help the technologist prepare a diagnostic slide. Unfortunately, some are time consuming but all tissues are important, and giving the pathologist a heads-up on the extra time required, makes the process less tense.

Surface decalcification

This is used when partially decalcified bone or unsuspected mineral deposits in soft tissue are found during block sectioning. This technique is done to prevent knife damage and scored tissue sections. After finding a calcification, the exposed tissue surface in a paraffin wax block is placed face down in 1% HCl, 10% formic or a proprietary acid solution for 15–30 minutes, rinsed with water to remove corrosive acids, the block wiped dry (to prevent ice crystals), cooled and re-sectioned. Extensive soaking may cause visible tissue swelling away from the block face and even though the tissue cuts more easily, the sections fall apart on the water bath.

The acid only removes a few micrometers of calcium from the tissue surface, and only a few sections can be cut after careful block re-orientation in the microtome. The first few sections may come off a little thicker than the microtome setting so pick up sections following those thicker sections. Another problem may be the calcified area pulls out of the block during microtomy. This can be avoided by sectioning slowly.

Poor fixation

This is the hardest problem to correct; if the bone is not fully fixed it cannot be decalcified or processed properly (Fig. 17.9). In this case, it is better to go back to the specimen and retrieve another piece, but if this is not possible, the following can be tried.

Firstly if the block looks like Fig. 17.9 follow the following protocol:

1. Remove all the paraffin wax from the block and around the tissue.
2. Place remaining tissue into a 56-60^{0}C oven and melt the paraffin wax from the tissue.
3. Place tissue into warm xylene to ensure all the paraffin wax is removed. Several changes and agitation may be needed.
4. Place tissue into 100%, then 95% alcohol for an hour each.
5. Place tissue cassette on the processor starting in 70% alcohol and re-process.

The 70% alcohol may help to fix the infiltration steps of the processing which will hold the tissue morphology in place during microtomy. It is impossible to try to place it in fixative again since the damage is done and hopefully the extended processing helps the microtomy. This is not recommended as it may not always reliable.

Secondly use the tape-transfer method which is described and illustrated later in this chapter (Sterchi & Eurell, 1990).

Poor processing

If a bone sample has been endpoint tested for completed decalcification but still appears chalky, mushy and crumbles out of the block during sectioning, then dehydration, clearing or paraffin wax infiltration may be incomplete. Blocks can be melted down and re-infiltrated with paraffin wax for up to 8 hours to see if this improves sectioning.

Adhesive tape methods

Adhesive tape methods or tape transfer methods are used to maintain the intact sections of undecalcified, double-embedded bone sections during microtomy. Two methods, one for undecalcifed bone embedded in MMA (Hardt, 1986) and the other for decalcified, paraffin wax-embedded bone (Sterchi & Eurell, 1990) are used for sectioning difficult blocks. The Sterchi tape-transfer method can be used on large and small, difficult to microtome tissues. Equipment which is needed in addition to the microtome is a 2-4 inch print roller, clear adhesive packaging tape (Scotch 3750), coated slides and a water bath (Fig. 17.10).

1. Trim (face) block.
2. Clean block face completely free of paraffin wax and dust.
3. Roll the tape onto the trimmed block face using the print roller to ensure complete coverage of tissue with the tape.
4. Slowly cut section while lifting the tape and section away from the knife.
5. The tape-section combination is then placed on a 42-45°C water bath with tissue side down.
6. Lift the section/tape off the water bath with a coated slide.
7. Using the print roller, press out all excess water from the slide and tape.
8. Carefully trim excess tape from the sides of slide.
9. Place a piece of heavy weight plastic wrap on top of the tape/tissue on slide.
10. Place the slide and wrap between pieces of thin wood or heavy plastic and clamp together to keep section flat. For drying, several slides can be stacked at one time as long as there is a piece of heavy duty plastic between each slide.
11. Dry in a 56-60°C oven overnight.
12. Remove the stack from the oven and carefully separate slides.
13. Remove the heavy duty plastic strips and place slides into a staining rack.
14. Place the slide rack into clean 100% xylene and allow the slides to soak until the tape falls off leaving the section 'transferred' onto the slide for subsequent staining.
15. Place the slides into clean xylene to remove any tape residue.
16. Stain the slides.

The tape-transfer method for large paraffin wax blocks is demonstrated in Figs. 17.11 & 17.12.

Staining methods for decalcified bone sections

Most routine soft tissue staining methods can be used without modification for staining decalcified bone sections. Acid decalcification, particularly when prolonged or used with a heat-producing method, e.g. microwave, sonication or electrolytic, can adversely affect the H&E and some special stains. When the temperature exceeds 37°C during decalcification, Giemsa staining may be too pink and the historical Feulgen stain for DNA will be negative because of excessive protein hydrolysis. Staining is successful after EDTA treatment, but the slower decalcification rate usually rules it out in favor of faster acid methods. H&E is still the primary stain used for most final diagnoses occasionally with the aid of special stains. Immunohistochemical staining is now an important aspect in disease diagnosis and is used frequently on decalcified bone tumors, bone marrow and cartilage. Most special stains used with bone sections are commercially available pre-mixed

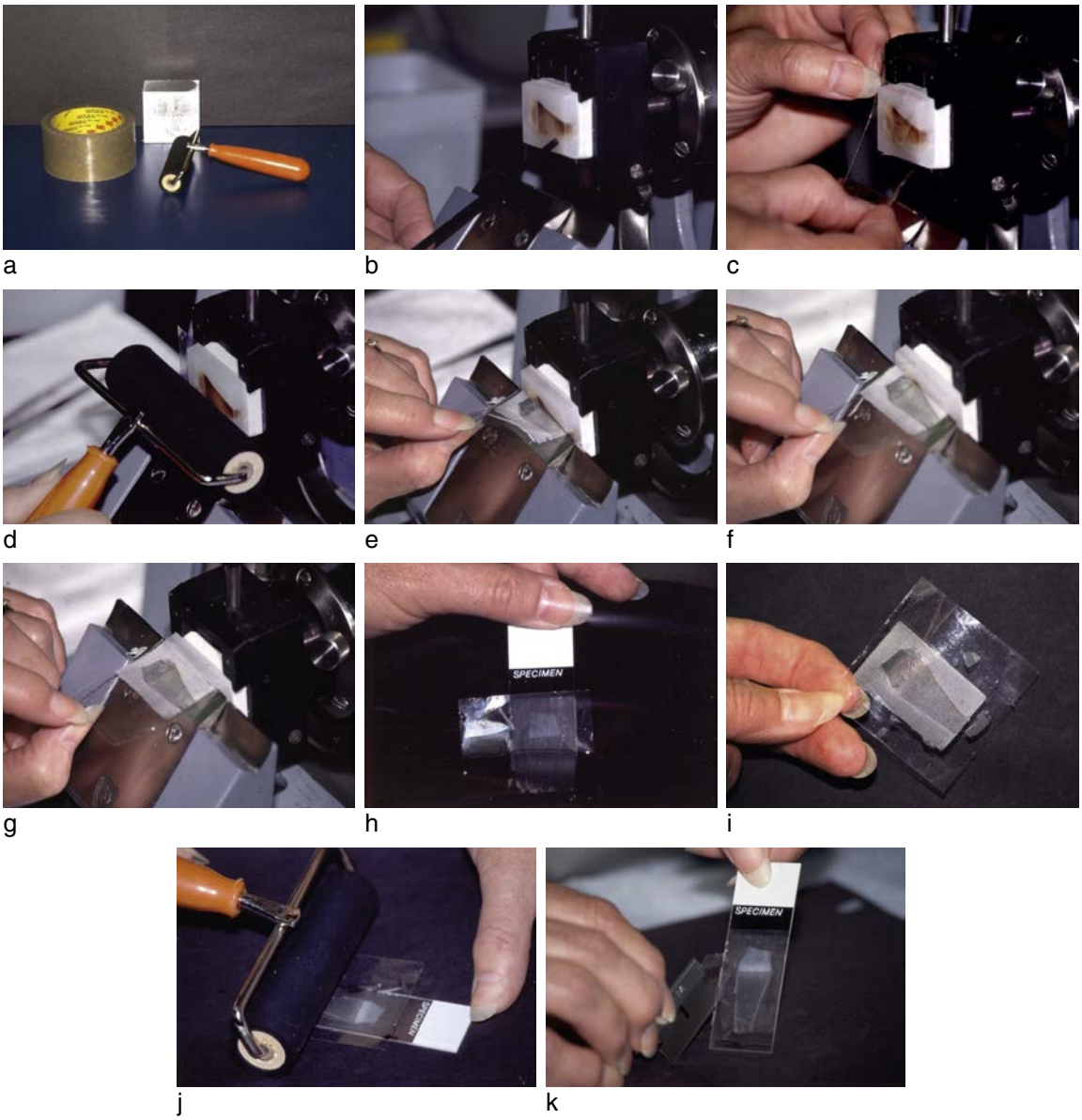


Fig. 17.10 Tape-transfer procedure for rotary microtome. (**a**) Equipment needed for procedure, (**b**) cleaning block face free of debris, (**c**) applying tape to block face, (**d**) pressing tape to entire tissue face using a print roller. (**e**) Start microtoming, (**f**) microtoming continuing while lifting tape and tissue away from knife, (**g**) completion of sectioning, (**h**) picking up tape/tissue section onto charged slide. (**i**) Tape and tissue section, (**j**) pressing tissue to slide and removing excess water, (**k**) trimming excess tape from slide.

and are more reliable to use than self-made stains. However, it is better to see the original ingredients/formulas in references to understand the mechanism and to help identify correct staining.

Hematoxylin and eosin (H&E)

When staining sections of properly decalcified tissue no modifications to the standard H&E techniques are required. There are several ways to

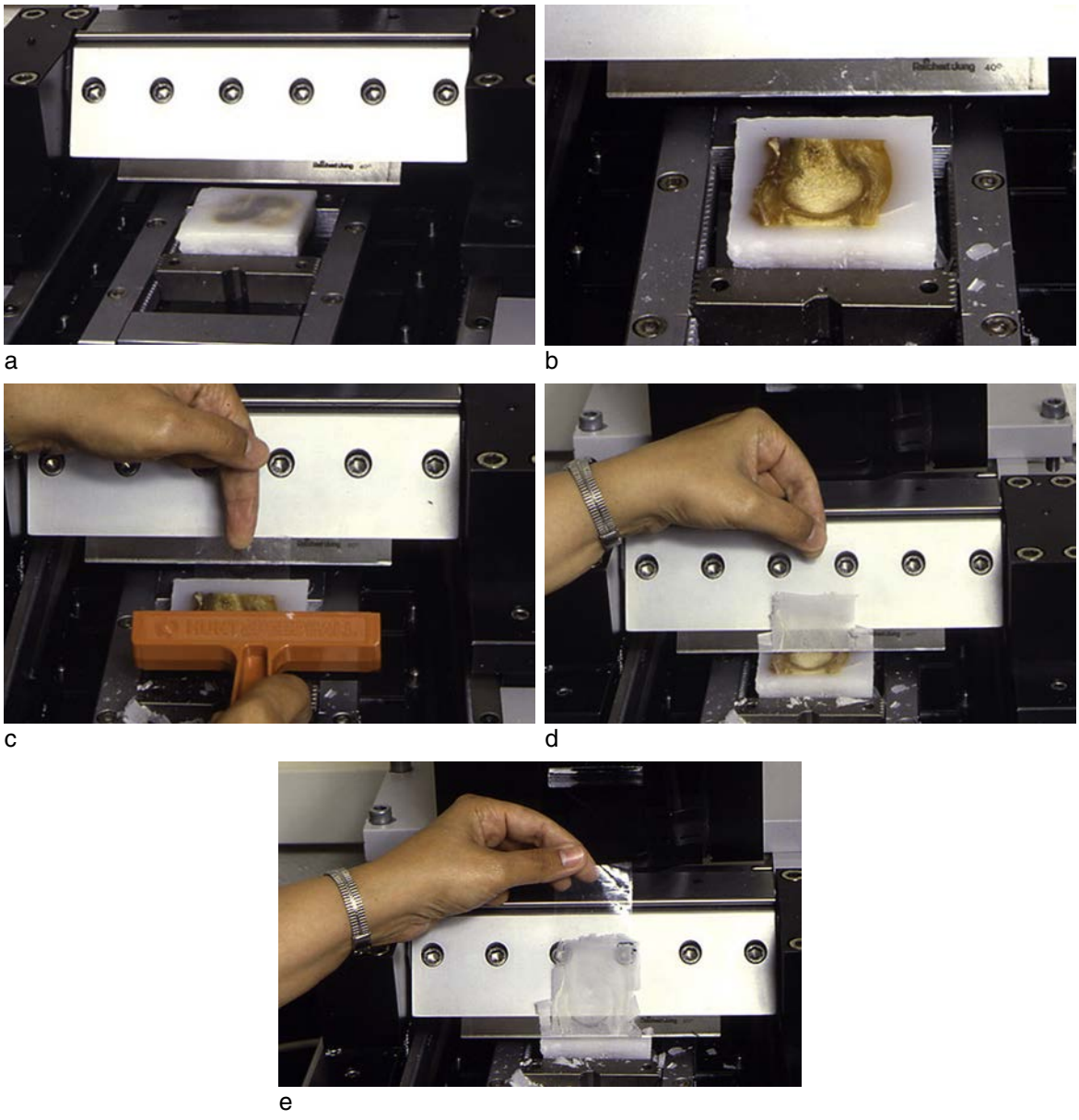


Fig. 17.11 Tape-transfer procedure for large paraffin blocks on a sliding microtome. (**a**) Block placed into microtome for facing, (**b**) faced and clean block, (**c**) applying tape to block face, (**d**) microtoming while lifting tape and section way from knife, (**e**) complete section on tape.

counteract weak nuclear staining damaged by acids and make the hematoxylin stain darker. Freshly prepared hematoxylins, particularly those which lose strength over time, e.g. Harris's, Gill II and III, often stain darker than solutions near their expiration date. The preference is to use a progressive hematoxylin since it does not require the differentiation steps a regressive hematoxylin does. Restoration of basophilic staining can be attempted by immersing a hydrated section in

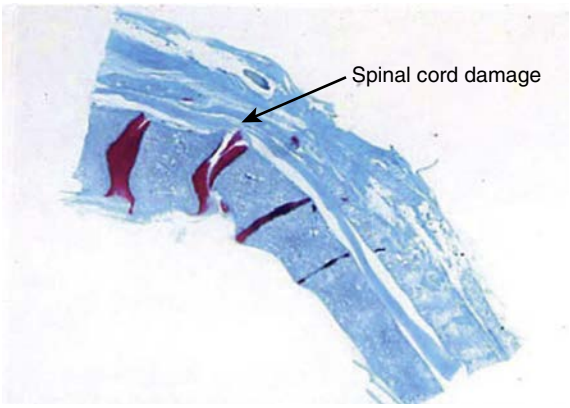


Fig. 17.12 Safranin O-fast green (SOFG)- stained portion of a penguin spine which was microtomed using the tape-transfer technique and demonstrates pinching of the spinal cord: the reason the penguin was unable to stand.

4–5% aqueous sodium bicarbonate for 10 minutes to 2 hours, rinsing well with water, and staining with hematoxylin. If using a regressive hematoxylin, the acid differentiation step can either be shortened to one or two fast dips in 0.5% acid alcohol, or this step can be eliminated entirely. When using a progressive hematoxylin, blueing solutions should be mild bases, e.g. Scott's tap water substitute or saturated lithium carbonate to avoid bone section loss caused by ammonia water 'blueing'. If poor hematoxylin staining is a persistent problem, it is recommended that the decalcification method be re-evaluated and appropriate changes made to avoid unreliable staining. Alcoholic eosin solutions (0.5–1%), often stain bone and surrounding tissues overly red, and staining time can be shortened from 1 minute to 30 seconds or even 10–20 rapid dips. Another excellent counterstain for differential staining of bone components is eosin Y-phloxine B.

In general, most hematoxylin solutions work well for staining bone, including mercury-free Harris's, Ehrlich's, Mayer's, Cole's, Gill II or III and many proprietary mixtures. Some workers prefer Ehrlich's hematoxylin to a more specific nuclear stain, e.g. Mayer's, for its ability to stain articular and growth plate cartilages a deeper blue to purple in contrast to the pink collagen and other tissues. In general, a hematoxylin solution can stain decalcified bone to show cement lines in Paget's disease of bone, new bone and rapidly formed or remodeled bone, provided the hematoxylin stains darkly enough. A good H&E can stain all cells and bone components including osteoid as long as care is taken to adjust the staining procedure to achieve optimal results.

Collagen stains

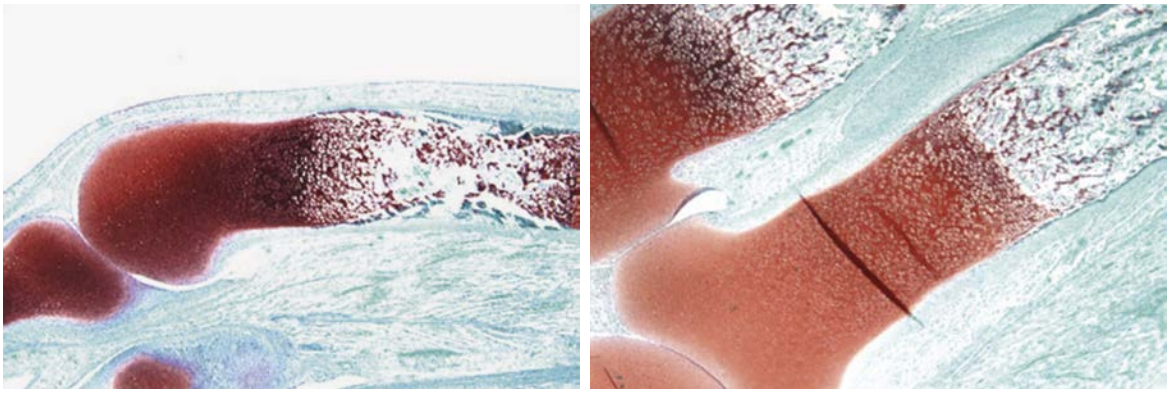
Collagen stains can be used to demonstrate mature and finer immature fibers in certain tumors and a fracture callus. van Gieson picro-fuchsin stains immature fibers a pale orange compared to the deeper red mature fibers. Masson's trichrome stain (see page 165) remains a standard, popular method to demonstrate collagen fibers in contrast to bone, cells and other soft tissues. The immature collagen fibers stain distinctly but are a paler blue or green compared with the darker stained mature fibers. Trichrome stained adult or mature bone often shows areas of blue or green staining with some bright red areas which frequently have no relationship to bone structure. Osteoid is usually stained with the aniline blue or light green fiber stains.

Polarized light microscopy may be more useful for positive identification of collagen than these stains since the finest fibers do not show distinct colors with routine light microscopy. Sirius red, which is commonly used for amyloid, is an excellent stain used to visualize collagen fibers under polarized light. A toluidine blue stain also demonstrates collagen fibers.

Cartilage and acid mucopolysaccharides

Cartilage can be stained to demonstrate mucopolysaccharides using various metachromatic staining methods, or the azure method by Hughesdon (1949) which is recommended for its selectivity and stability.

The critical electrolyte concentration method of Scott and Dorling (1965) provides a more precise identification of acid mucopolysaccharides in cartilage. They used 0.05% 8GX alcian blue in pH 5.8



a b

Fig. 17.13 SOFG-stained fetal (**a**) finger joint and (**b**) ulna and radius. Both **a** and **b** demonstrate a high concentration of chondrocytes near the articular ends.

acetate buffer containing 0.4-0.5 M magnesium chloride to stain these strongly sulfated mucopolysaccharides blue. Another useful method for showing articular cartilage degradation of ground substances in arthritic and other diseases is safranin O-fast green (SOFG) (Rosenberg, 1971). This stains the cartilage varying shades of red and is seen well in developing bones (Fig. 17.13). Toluidine blue O 0.1-1% aqueous solution is also commonly used to stain NBF-fixed cartilage. One should be aware that EDTA, as well as some fixatives and acid decalcifiers, extract proteoglycans and can result in weak cartilage staining by safranin O and possible false negative quantitative results (Callis & Sterchi, 1998).

The positive red periodic acid-Schiff (PAS) reaction demonstrates mucopolysaccharides in new bone, calcifying cartilage and glycogen in some early osteoblasts. PAS assists in the diagnosis of some mucinous metastatic tumors and primary tumors with glycogen when a diastase digestion method helps make a more precise identification of a poorly differentiated primary tumor. The PAS reaction is not affected by decalcification, but prolonged treatment with strong acids should be avoided. Reticulin staining, the silver impregnation of reticulin fibers, helps in the diagnosis of bone tumors, tumor metastasis to bone and myelofibrosis. Reticulin staining is not affected by decalcifying agents although the ammoniacal solutions can cause a section to release from the slide, necessitating the use of a stronger section adhesive.

Bone canaliculi

Osteocytes and their lacunae are large enough to be easily identified in most preparations, including H&E-stained paraffin wax sections of decalcified bone. Fine canaliculi radiating from lacunae are not easily seen in H&E-stained sections, but are well demonstrated with a modified Holmes' silver impregnation method on buffered, formic acid decalcified, paraffin wax-embedded bone sections (Taylor et al., 1993).

The major problem in demonstrating canaliculi is the attempt to show spaces, too fine for identification by routine staining of the surrounding bone. It is necessary to fill the spaces with a substance which appears dark against a lighter or unstained background. The simple 'air injection' method (Gatenby & Painter, 1934) using undecalcified ground sections dried and mounted in hot, melted Canada balsam traps air inside the canaliculi. These look like black threads against the unstained bone and the balsam but, if sections are too thin, or the balsam too fluid, the air will be displaced. This method is not commonly used in clinical facilities unless specializing in orthopedics. Today celloidin is used rarely; its popularity

has reduced because of safety issues but it was used in the past for embedding and attaching tissue sections to slides.

Immunohistochemistry (IHC)

Diagnostic immunohistochemical staining frequently uses decalcified bone sections embedded in paraffin wax (e.g. bone marrow biopsies, tumors and cartilage), and uses the same staining methods and materials as for soft tissue IHC. Care must be taken to fix bone specimens properly and decalcify with the least damaging agent in the shortest possible time in order to protect antigens from the damaging effects of acids. Immunostaining is possible on 2 μm thick methyl methacrylate sections after complete removal of the plastic with warm xylene and a pressure cooker antigen retrieval method (Hand & Church, 1998). Glycol methacrylate (GMA) cannot be removed and may inhibit adequate antibody or immunoglobulin penetration to the antigenic sites. It is suggested that the bone be stained using DAB chromogen after fixation and before GMA embedding. Counterstains work well after microtomy when processed in this manner and it does not diminish or wash out the chromogen.

Preparation of mineralized bone

Sections demonstrating bone mineral and its relationship to the unmineralized components of bone must be prepared by methods which do not interfere with the mineral substance, i.e. undecalcified bone sections. Mineralized bone must be cut with tungsten carbide-tipped knives and needs special, hard support to avoid cracked or crumbling tissue sections. Paraffin wax is too soft and fails to match the hardness of bone or provide the strong, solid support needed to prevent fragmented, mineralized sections.

Acrylic resins and plastics are now widely used and the preferred embedding media for undecalcified bone; their use has revolutionized how this tissue is examined. Frozen sections provide some support of cancellous bone, but the bone itself tends to look damaged and somewhat fragmented even though a diagnosis could be made from the soft tissue components. These methods are described in Bancroft et al. (2012).

The main reasons for embedding mineralized bone in plastics are (see Chapter 8):

- Mineralization can be measured allowing the osteoid to be distinguished from the mineralized components.
- Cellular morphology is undisturbed (osteoclasts, osteoblasts, fibroblasts, and hematopoietic or tumor cells).
- Trabecular mineralization fronts, cartilage and other bone components are undisturbed.
- Fluorescent labeling is retained.
- For histomorphometric analysis.

Morphometry of bone

Histomorphometric analysis can be done with manual, semi-automated or automated methods. A manual method could include a standard microscope with eyepiece reticules, digitized tablets (image display), image storage and a computer for data storage and output. Increasingly, modern automated computerized image analysis systems with a video camera, a screen with screen grid for area counting, and software designed specifically for bone work are being used to reduce the time needed for measurements and calculation of the final results.

A standardized, generally universal system of nomenclature, symbols and units for bone histomorphometry was summarized by Parfitt (1988) and it is recommended that users be familiar with and use this system. It is a terminology list of primary measurements for volume, surface, thickness, mineralization rate, formation rate etc. Basic measurements are confined to trabecular bone and are:

- Trabecular bone volume and surface.
- Eroded (resorption) surface.
- Osteoid surface.
- Mineralized surface.
- Osteoid thickness.

- Wall thickness (of new bone layers at formation site).
- Mineral apposition rate (calcification rate) (Recker, 1983).

Calculations are made from collected data (Parfitt et al., 1987) and results correlated to the various diseases. Parfitt explains how area measurements are numerically equated with volume measurements. These equations are still used today and performed by computer software programs. The derivation of some of these values is shown below.

Bone volume (%)

$$\text{Osteoid volume } (\%) = \frac{\text{area of osteoid}}{\text{area of trabeculae and marrow space}}$$

$$\text{Osteoid surface } (\%) = \frac{\text{length of trabecular surface covered by osteoid}}{\text{total length of trabecular surface}}$$

$$\text{Osteoid index } (\%) = \frac{\text{osteoid volume}}{\text{osteoid surface}}$$

$$\text{Resorption surface } (\%) = \frac{\text{length of trabecular surface occupied by lacunae}}{\text{total length of trabecular surface}}$$

There are many other derived parameters used to describe bone dynamics described by Frost (1983).

Histomorphometric values for normal males and females with relation to age and values for the various diseases, compared to age and sex matched normal controls, have been published and are useful as reference guidelines (Melsen et al., 1983). It is important to be aware of pitfalls in techniques for bone morphometry; these are well discussed by Recker (1983). An example of a problem is with serial biopsy evaluation of severe Paget's disease where known variations occur from site to site, and even within the same bone. It is important that each laboratory establish its own set of normal values along with careful standard operating procedures (SOPs) for sample preparation, staining techniques and microscopy used for bone morphometry. If standardized stains or magnification are not used, measurements made using different stains can result in different values for the same biopsy. A standardized magnification must be used to estimate surface values, otherwise higher values are produced with increased magnification as finer surface convolutions are resolved. Detailed discussions of bone morphometry can be found in Recker (1983), Jee and Parfitt (1980) and a useful review by Revell (1986).

Microcomputed tomography (microCT)

This is a tool which allows the micro-analysis of bone and tissue without destruction of the sample. MicroCT works on the same principles as clinical CT. The samples are fixed and kept in a 70% alcohol solution during microCT. A radiation source then releases X-rays through a sample and the exiting X-rays are collected on the detector. In many microCT units the sample rotates while projection images are collected at various angles. Generally, up to a thousand projections are taken at greater than 190 degrees around the sample. Reconstruction then takes the planar projections and compiles them into a three-dimensional (3D) image which can be manipulated in all three orthogonal planes. Changing the distance of the sample from the X-ray source and detector, or the number of projections can influence the resolution of the scan (Hsieh, 2003).

Different tissue types block or 'attenuate' X-ray radiation at varying degrees. This attenuation property of tissue of differing densities creates the contrast in the image. Bone has a higher density than soft tissue, hence its ease of visualization with CT. With a 3D image one can perform geometric analysis and quantify trabecular size, number, thickness, spacing and connectivity, along with bone mineral density and content (Abe et al., 2000). The use of contrast agents allows for the production of 'virtual histology' of a tissue (Johnson et al., 2006) (Fig. 17.14).

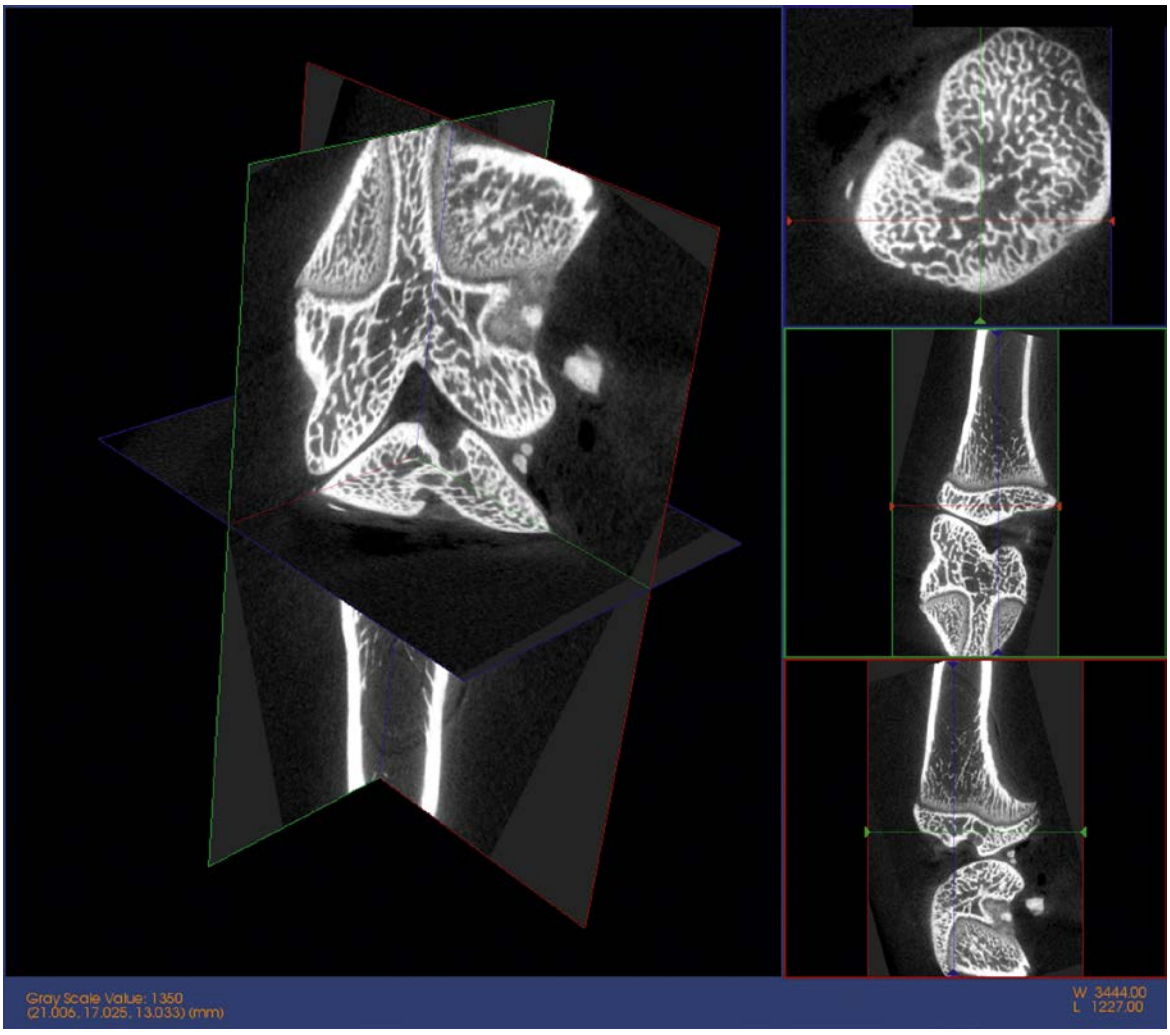


Fig. 17.14 Example of a specimen CT scan showing different dimensional measurement capabilities.

Acknowledgments

The author would like to thank the following for their contributions to this chapter: Gayle Callis, for tape transfer photographs; and Chris Bull, for his expertise in CT and providing Fig. 17.14.

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18 Neuropathology and muscle biopsy techniques

J. Robin Highley • Nicky Sullivan

Introduction

In neuropathology, as in most other areas of histological practice, hematoxylin and eosin (H&E) remains the most useful and commonly used stain, as it demonstrates most cell types well with good detail. As with other histological disciplines, neuropathology relies more on immunohistochemistry and cytogenetics and less on the more capricious and obscure preparations of the past. Nonetheless, a number of reliable and useful tinctorial and metal-based stains remain in common usage.

Neuropathological practice increasingly stresses the importance of molecular and cytogenetic aspects of disease (Brandner & von Deimling, 2015). This is most clearly illustrated by the 'layered' approach to diagnosis in the recent WHO tumor classification (Louis et al., 2016) whereby morphological, immunohistochemical and cytogenetic data are built up into a final 'integrated' diagnosis. Such genetic techniques include in situ hybridization, Sanger and pyrosequencing and 'next generation' sequencing (Sahm et al., 2016). Currently, many of these are performed in supraregional cytogenetics laboratories but, as these methods increase in number and importance, it is essential that histology staff become conversant with them.

Muscle biopsy techniques are included in this chapter as these methods tend to be performed in the areas of the laboratory designated for neuropathology.

The components of the normal central nervous system

The nervous system can be subdivided into the central, peripheral and autonomic nervous systems. This chapter will concern itself principally with the central nervous system and secondarily with the peripheral nervous system and muscle. The principal components of the central nervous system are:

- Neurons
- Glial cells
- Meninges
- Blood vessels.

The neuron is an excitable cell which is responsible for processing and transmitting information. Neurons communicate with each other via intercellular interfaces called synapses. At the synapse, an electrical impulse in the presynaptic neuron causes it to release a chemical transmitter which diffuses across a narrow gap to influence the electrical activity of the postsynaptic target neuron. Neurons have several components (Fig. 18.1):

- The cell body (or 'soma'), containing various subcellular organelles responsible for the metabolic upkeep of the cell.
- The nucleus, which resides in the cell body and is the site of storage of the cell's genetic code in DNA.
- The dendritic tree, a complex of cell processes responsible for receiving synaptic inputs from other neurons.
- The axon, an elongated fiber which transmits electrical impulses away from the soma to synapses with either other neurons or muscle fibers. This may be a meter or more in length in the case of the lower motor neurons which reside in the lower (lumbar) spinal cord and innervate the muscles of the lower leg.

Two naturally occurring pigments may be observed to accumulate in the brain with age, lipofuscin

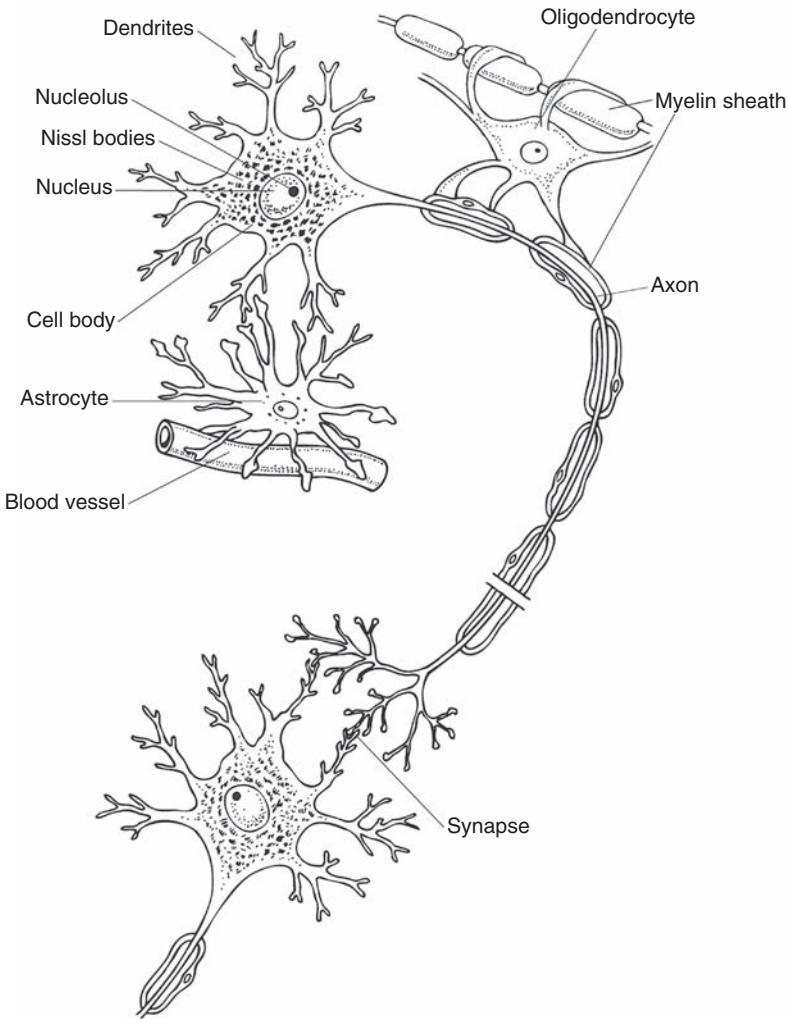


Fig. 18.1 Diagram showing a neuron with its component parts together with other cells of the central nervous system. (Courtesy of Patrick Elliott of the Medical Illustration Department, Royal Hallamshire Hospital, Sheffield, UK).

and neuromelanin. Both are believed to represent cellular waste products. Lipofuscin is a yellow-brown, autofluorescent, granular substance composed of peroxidized protein and lipids. It is seen in larger neurons, such as the lower motor neurons of the spinal cord and pyramidal cells of the hippocampus in Alzheimer pathology. Large amounts of lipofuscin-like pigment also accumulates in inherited neuronal ceroid lipofuscinoses, of which Batten's disease is the most common (Goebel & Wisniewski, 2004). Neuromelanin is most commonly seen in the cytoplasm of neurons of the substantia nigra and the locus ceruleus. It is the cause of the macroscopic pigmentation of these structures. Under the microscope, it is a dark brown, granular material which is believed to be the by-product of oxidative metabolism of catecholamines (Sulzer et al., 2008).

Glial cells are the support cells of the nervous system. They are diverse in nature, and the principal types and their functions follow.

- Astrocytes have a number of functions. Firstly, they maintain the extracellular ion and neurotransmitter balance. Secondly, they are involved

in repair and scarring responses to brain damage and finally astrocytes form part of the blood–brain barrier, protecting the brain from harmful blood-borne substances.
- Oligodendroglia form myelin, a phospholipid sheath around nerve axons which enhances the speed of conduction of impulses.
- Ependymal cells line the ventricles of the brain and central canal of the spinal cord.
- Microglia are the native immune cell of the nervous system of monocyte/macrophage lineage.

Neuropil is a term used to denote the feltwork of neuronal processes in which neuron cell bodies reside. Central nervous system tissue is classically subdivided into gray matter, containing the majority of neuronal cell bodies and little myelin, and white matter, which is predominantly formed of myelinated axons and few neurons.

The meninges form three layers of protective covering over the brain. The outer layer, beneath the skull, is the dura mater; a tough, fibrous membrane. The arachnoid mater is a more delicate, fibrillary covering which lies inside the dura mater and is more closely adherent to the brain surface, but it does not invaginate into the surface infoldings of the brain (or sulci). The pia mater is the most delicate covering. It is closely apposed to the brain surface, following its contours down into the depths of sulci.

Techniques for staining neurons

Tinctorial stains for Nissl substance

Hematoxylin and eosin (H&E) preparations demonstrate the most important features of neurons. However, Nissl preparations are also popular for examining the basic architecture of neural tissue and its components. These are often combined with the luxol fast blue myelin stain. Granules of Nissl substance are found in the cell body (Fig. 18.1) and correspond to rough endoplasmic reticulum. They are basophilic due to the associated nucleic acid (Palay & Palade, 1955). Many basic dyes e.g. neutral red, methylene blue, azure, pyronin, thionin, toluidine blue and cresyl fast violet stain Nissl substance. Variation in the stain used, pH and degree of differentiation allow preparations to label either Nissl substance alone, or Nissl substance in combination with cell nuclei.

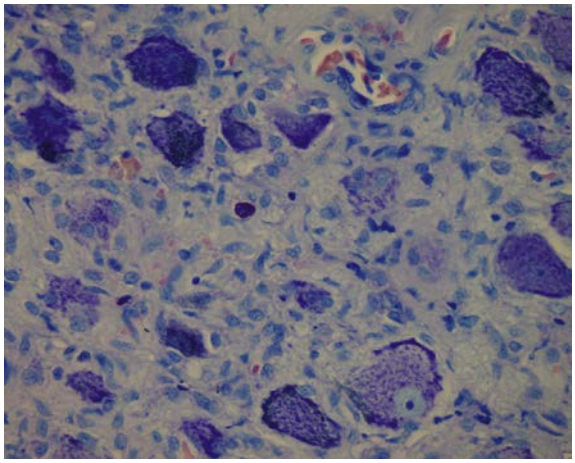


Fig. 18.2 Anterior horn cells in spinal cord. Notice their large size and the prominent nucleolus. Paraffin section, stained with toluidine blue. Similar results can be obtained with cresyl fast violet.

Motor neurons generally have coarse ('tigroid') Nissl substance, and regions such as the anterior horns of the spinal cord, where these cells are abundant, are good tissues to use when learning these stains (Fig. 18.2). For paraffin wax-embedded sections of formalin-fixed tissue, the cresyl fast violet stain is reliable and relatively straightforward. As such it is by far the most commonly used Nissl preparation. Toluidine blue may also be used, whilst Einarson's gallocyanin method, being more suited to alcohol-fixed tissue, is largely unused (Kellett, 1963).

Cresyl fast violet (Nissl) stain for paraffin wax sections

Fixation

Alcohol, Carnoy's or formalin.

Sections

Paraffin wax, 7–10 µm or 25 µm (see Note b).

Preparation of stain

Cresyl fast violet	0.5 g
Distilled water	100 ml

Differentiation solution

Glacial acetic acid	250 µl
Alcohol	100 ml

Method

1. Dewax sections and bring to water.
2. Cover with filtered cresyl fast violet, stain for 10–20 minutes.
3. Rinse briefly in distilled water.
4. Differentiate in 0.25% acetic alcohol until most of the stain has been removed (4–8 seconds).
5. Briefly pass through absolute alcohol into xylene and check microscopically.
6. Repeat steps 4 and 5 if necessary, giving less differentiation when repeating.
7. Rinse well in xylene and mount in DPX.

Results

Nissl substance	purple/dark blue
Neurons	pale purple/blue
Cell nuclei	purple-blue

Notes

a. If only Nissl substance is required to be demonstrated, the stain is acidified with 0.25% acetic acid.

b. Estimation of cortical neuronal density is made on 25 µm thick sections.

c. The cresyl violet method can be used as a counterstain when demonstrating myelin with the Kluver and Barrera method (page 312).

Immunohistochemistry of neurons

The protein targets of antibodies used in immunohistochemical (IHC) preparations for the demonstration of neuronal elements can be classified into four main groups:

1. **Neuronal cytoskeletal proteins.** *Neurofilaments* (NF) are intermediate filaments expressed by mature neurons. They are composed of protein subunits and are classified by molecular weight (light, medium and heavy) into NF-L, NF-M and NF-H which may be variably phosphorylated (Gotow, 2000). Antisera raised against different neurofilament proteins in different states of phosphorylation are available. NF-H in particular, and NF-M to a lesser extent, are normally unphosphorylated in the neuronal cell body, but become phosphorylated in the axons. Thus, antibodies to phosphorylated NF-H mark axons but not cell bodies in normal nervous system tissues. Antibodies to non-phosphorylated neurofilament will label neuronal somata (Trojanowski et al., 1986). *Microtubule-associated protein 2 (MAP-2)* is a protein involved with microtubule assembly and is expressed by neurons in dendrites and cell bodies (Maccioni & Cambiazo, 1995; Shafit-Zagardo & Kalcheva, 1998). It is often used as a marker of neuroepithelial differentiation (Blumcke et al., 2004; Wharton et al., 2002).
2. **Cytoplasmic proteins.** *PGP9.5* and *neuron-specific enolase (NSE)* are strongly expressed in neurons and can reliably be labeled by commercially available antisera. Unfortunately, they are not specific for neuronal cells, making interpretation tricky. In truth, many pathologists find them of little use.
3. **Neuronal nuclear proteins.** *NeuN* is a neuron-specific DNA binding protein, which starts to be expressed around the time of initiation of terminal differentiation of the neuron (Mullen et al., 1992). Antibodies to NeuN label neuronal nuclei and neuronal components of other tumors (Edgar & Rosenblum, 2008). In the context of neuro-oncology however, NeuN, lacks specificity, being expressed to a variable degree in a diverse range of primary brain tumors. Therefore, NeuN is best used as part of a panel of antibodies in the investigation of clear cell primary brain tumors, but is of limited use for other tumors (Preusser et al., 2006).
4. **Proteins associated with neurosecretory granules**. Antisera to these proteins can be useful to establish neuronal and neuroendocrine differentiation (Koperek et al., 2004; Takei et al., 2007). *Synaptophysin* is a membrane glycoprotein component of presynaptic neurosecretory vesicles. The cell body of normal neurons is usually unstained by synaptophysin (Fig. 18.3), resulting in early claims that cell body labeling was a feature of neoplastic neuronal cells which differentiated them from native neurons (Miller et al., 1990). However, it is now evident that a population of normal neurons also show cell body labeling which detracts from the use of this feature as a diagnostic marker (Quinn, 1998). Synaptophysin is a useful marker of neuroendocrine differentiation and so also stains cells in metastatic neuroendocrine tumors (Wiedenmann et al., 1987).

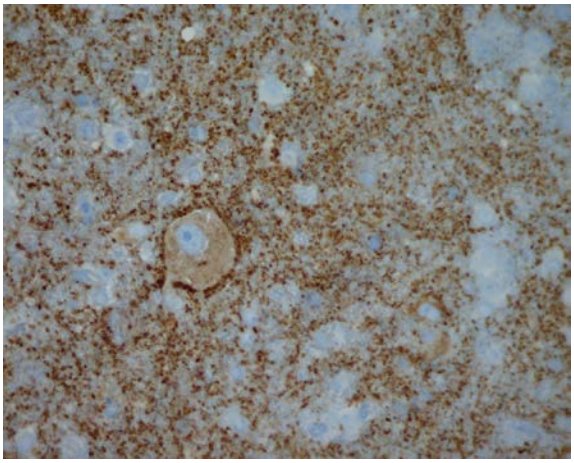


Fig. 18.3 Large neuron from an area of cortical dysplasia stained with synaptophysin. Note intense staining within the neuropil and weak cytoplasmic staining.

Chromogranin A is a protein of the dense core matrix of neurosecretory granules, and antibodies to it can be used to identify cells containing dense core vesicles (Nolan et al., 1985). It is used predominantly to elucidate neuroendocrine differentiation in tumors.

Techniques for staining axons and neuronal processes

Immunohistochemistry has largely replaced the old silver preparations for the demonstration of axons as it is reliable and produces adequate results for diagnostic neuropathology with ease. However, for formal quantitation of axons, many still find that Palmgren's method is superior to neurofilament IHC (Chance et al., 1999). This technique has classically been used for staining axons of the peripheral nervous system, but it is also excellent for staining central nervous system axons. Palmgren's method uses potassium nitrate to suppress reticulin staining. It is considered that using the Palmgren method, cresyl violet preparations and IHC for neurofilament and MAP-2, there is no longer a requirement for the older silver preparations, e.g. Bielschowsky (Bielschowsky, 1902) and Marsland (Marsland et al., 1954).

Silver techniques require great care and attention to detail such as clean glassware and pure distilled water for a successful outcome. Stock solutions should be well maintained and not more than a few months old, in some cases less than a week.

Modified Palmgren's method for nerve fibers in paraffin wax-embedded material (Palmgren, 1948)

Fixation

Formalin fixed tissue.

Sections

Paraffin wax sections 6–10 µm. Sections should be on coated slides.

Preparation of solutions

Acid formalin

Concentrated formaldehyde (40% w/v)	25 ml
Distilled water	75 ml
1% nitric acid	0.2 ml

Silver solution

30% silver nitrate	25 ml
20% potassium nitrate	25 ml
5% glycine	0.5 ml

Reducer

Pyrogallol	10 g
Distilled water	450 ml
Absolute ethanol	550 ml
1% nitric acid	2 ml

Fixing bath

5% sodium thiosulfate.

Method

1. Take sections to distilled water.
2. Treat with acid formalin for 5 minutes.
3. Wash in three changes of distilled water for 5 minutes.
4. Leave in filtered silver solution for 15 minutes at room temperature.
5. Without rinsing, drain the slide and flood the section with reducer which has been heated to 40–45°C. Rock the slide gently and add fresh reducer. Leave for 1 minute. A beaker placed on a hot plate is useful for this stage.
6. Wash in three changes of distilled water. Examine microscopically and, if necessary, repeat from step 4, reducing the time in the silver solution and decreasing the temperature of the reducer to 30°C.
7. Wash in distilled water.
8. Fix in 5% sodium thiosulfate for 5 minutes.
9. Wash in tap water.
10. Dehydrate in alcohol. Clear and mount in DPX.

Result

Nerve fibers	brown or black

Notes

a. In the original method the silver solution contained 5% acetic acid rather than 5% glycine. Glycine must be made up fresh prior to use as it is only stable for approximately one week. However, the Palmgren silver solution, once made up, is stable for several weeks.

b. The silver incubation time may need to be increased for tissues which have had a short formalin fixation time, you should see a slight yellow tinge to the tissues when the optimum time has been reached. The reducer keeps for several months.

c. The original method stated that the reducer had to stand for 24 hours before use, but this is not the case. The reducer will darken with time, changing from pale yellow to dark amber. It is important that at the reduction step the slides are gently agitated to ensure an even reduction of the tissue; if the sections are not dark enough, they can be rinsed in distilled water and steps 4–6 repeated but with a shorter time in the silver solution.

d. The hotter the reducer, the faster the reduction will take place and it may well be uneven, leading to suboptimal preparations. Sections can be toned using gold chloride prior to fixing, which is an optional step.

e. The original method used an intensifying step prior to fixing using aniline. Some have found this to be of little value. The method was originally designed for use with paraffin wax sections. However, it can be applied to cryostat sections which have been pretreated with 20% chloral hydrate overnight prior to carrying out the Palmgren method.

Examination of axons in peripheral nerves in diagnostic neuropathology now relies largely on toluidine blue stained, semi-thin resin-embedded tissue. Capricious techniques such as Eager's method for detecting degenerating axons are no longer in use (Eager et al., 1971).

The Golgi preparation and its variants are excellent for the visualization of the three-dimensional nature of the neuron and its dendritic processes, but modern diagnostic neuropathology practice has no requirement for these. Golgi techniques are occasionally used in research (e.g. Garey, 2010), although new antisera are increasingly allowing IHC indices of these aspects of cell morphology. It is suggested that the interested reader consider the Pugh and Rossi modification for use on paraffin wax-embedded tissue (Pugh & Rossi, 1993) if a Golgi stain is to be attempted.

Myelin

Myelin forms an electrically insulating sheath around axons. It is approximately 80% lipid and 20% protein and is formed from sheet-like processes of glial cells concentrically wrapped multiple times around the axon. This greatly improves the speed and efficiency of impulse conduction along the axon. Myelin is formed by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. A single oligodendrocyte may myelinate multiple axons in its vicinity, whereas a single Schwann cell only myelinates a single segment of a single axon. Loss of myelin, as is seen in multiple sclerosis in the central nervous system or in Guillain-Barré syndrome in the peripheral nervous system, can be severely debilitating.

Modern tinctorial stains for myelin are simple and reliable and can be performed on formalin-fixed paraffin wax-processed tissue. Many can be combined with a Nissl stain to demonstrate neuronal localization. We also find that the myelin staining is more intense and satisfactory when combined with Nissl staining than without. Older methods may give more even and consistent staining, but are considerably more time consuming and have fallen from use (Loyez, 1910; Weigert, 1904; Weil, 1928). Luxol fast blue, solochrome cyanine and IHC preparations are now favored. Many antisera are used as markers of myelination, the most useful being myelin basic protein, proteolipid protein and myelin associated glycoprotein (Itoyama et al., 1980a, 1980b; Lindner et al., 2008; Ludwin & Sternberger, 1984). Given the reliability and simplicity of the tinctorial stains, IHC myelin markers are rarely used in routine diagnostic neuropathology and remain the preserve of research laboratories. Immunohistochemistry for S-100 is useful in the diagnosis of tumors derived from

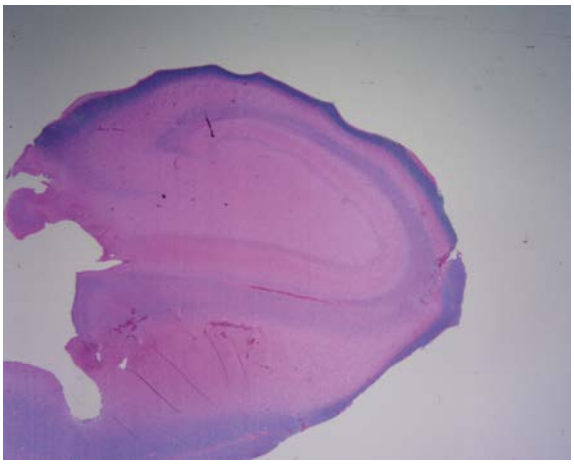


Fig. 18.4 Macro section of a human hippocampus demonstrating geographical variation in myelin content, stained with luxol fast blue.

Schwann cells both in the central nervous system and peripherally (Rodriguez et al., 2012).

Luxol fast blue is a copper phthalocyanine dye which is employed in myelin staining of paraffin wax-processed tissue (Kluver & Barrera, 1953). This can be combined with cresyl violet or hematoxylin to outline cellular architecture (Fig. 18.4) or with periodic acid-Schiff (PAS) to demonstrate myelin degradation products in demyelinating disease in central nervous system tissue only.

Luxol fast blue stain for myelin with cresyl violet counterstain (Kluver & Barrera, 1953)

Fixation

Formalin.

Sections

Paraffin, 10–15 µm.

Preparation of solutions

Luxol fast blue

Luxol fast blue	1 g
Methanol (absolute)	1000 ml
10% acetic acid	5 ml

Mix reagents and filter.

Cresyl violet stock solution

Cresyl violet	0.5 g
Distilled water	100 ml

Acidified cresyl violet solution

Cresyl violet stock solution	100 ml
10% acetic acid	0.8 ml

Filter before use.

Method

1. Take sections on slides to 95% alcohol (*not* water).
2. Stain in luxol fast blue solution for 2 hours at 60°C, or 37°C overnight.
3. Wash in 70% alcohol.
4. Wash in tap water.
5. Differentiate in 0.1% lithium carbonate solution until the gray and white matter are distinguished. This may be more easily controlled by using 0.05% lithium carbonate followed by 70–95% alcohol instead.
6. Wash in tap water.
7. Check differentiation under the microscope. Repeat step 5 if necessary.
8. Stain in cresyl violet solution for 10–20 minutes.
9. Drain sections and transfer to 70% alcohol. Avoid placing the section in water at this stage as the cresyl violet staining loses some of its intensity. Gently agitate the sections; the cresyl violet dye will flood out. The 70% alcohol differentiates the cresyl violet stain. Optimally, the cresyl violet should be removed, leaving the cell bodies and Nissl clearly visible. Do not over-differentiate; the 70% alcohol will take out the cresyl violet and to a certain extent the luxol fast blue. The cresyl violet counterstain will deepen the color of the luxol fast blue-stained myelin from turquoise to a deep blue.
10. Dehydrate, clear in xylene and mount in DPX.

Result

Myelin	blue
Nuclei and Nissl substance	violet/pink

Notes

a. If the section is over-differentiated with lithium carbonate/alcohol, the section can be restained with the luxol fast blue and then differentiated to obtain the optimum staining result. This may apply to tissues which have very low amounts of myelin e.g. baby/neonatal brains. These tissues can be very challenging in achieving optimum staining. Unfortunately, once the cresyl violet counterstain has been applied the over-differentiation cannot be rectified.

b. Some histologists prefer to differentiate the cresyl violet using 0.25% acetic acid in 100% alcohol.
c. The use of thick sections is important for the visualization of myelin tracts.
d. Other counterstains may be used such as neutral red. This will result in the myelin appearing purple/blue due to a slightly different color balance, and is a matter of personal preference. The solochrome cyanine stain is a simple and rapid technique for demonstration of myelin in both the central and peripheral nervous systems.

Solochrome cyanine technique for myelin in paraffin wax sections (Page, 1965)

Fixation

Formalin.

Sections

Paraffin wax, 6–10 µm. Cryostat section, 10 µm.

Preparation of solution

Solochrome cyanine RS	0.2 g
Distilled water	96 ml
10% iron alum	4 ml
Concentrated sulfuric acid	0.5 ml

Method

1. Take sections to water.
2. Stain for 10–20 minutes at room temperature.
3. Wash in running water.
4. Differentiate in 5% iron alum until all the nuclei are unstained. Wash frequently in distilled water, and examine microscopically.
5. Wash in running tap water.
6. Counterstain if desired.
7. Dehydrate, clear and mount.

Result

Myelin sheaths	blue

Notes

a. The staining solution keeps well.
b. Neutral red, neutral fast red, or van Gieson can be used for counterstaining.

Myelin loss may occur in a region of brain damaged by any of a number of processes, e.g. trauma, neoplasia, multiple sclerosis or toxic insult. It may also occur secondary to the loss of axons emanating from any damaged brain region. In modern practice, degeneration of myelinated tracts is most commonly demonstrated by showing loss of normal myelin staining by either luxol fast blue or solochrome cyanine preparations, or by showing a microglial reaction using CD68 IHC (Ince et al., 2003).

The neuroglia

This term refers to the supporting cells of the central nervous system and comprises ependymal cells, astrocytes, oligodendrocytes and microglia. As is becoming a recurrent theme, IHC is increasingly replacing tinctorial stains for their identification.

Ependymal cells

These cells are epithelioid and line the ventricles of the brain and the central canal of the spinal cord. They are easily located with conventional stains such as H&E and IHC for glial fibrillary acid protein (GFAP), vimentin and S-100. Immunohistochemistry for epithelial membrane antigen (Hasselblatt & Paulus, 2003; Uematsu et al., 1989) labels both normal and neoplastic ependymal cells, whilst cytokeratin markers are negative.

Astrocytes

These cells have multiple, fine processes and in their reactive state are 'star-shaped', hence the name. On standard H&E sections, only the nucleus of resting astrocytes is distinct, as the cell body cannot be discerned from background neuropil. These nuclei are slightly larger with more open granular chromatin than those of the more compact oligodendrocyte. Modern neuropathology relies most heavily on GFAP (Fig. 18.5) IHC for the demonstration of astrocytes, although antibodies to S-100, αB-crystallin and glutamine synthetase may also be used.

Astrocytes are principally classified into protoplasmic and fibrous forms. These are similar in function, but protoplasmic astrocytes have shorter, thicker, highly branched processes and are generally found in the gray matter; fibrous astrocytes have longer, thinner, less-branched processes and usually reside in the white matter. Astrocytic reactions

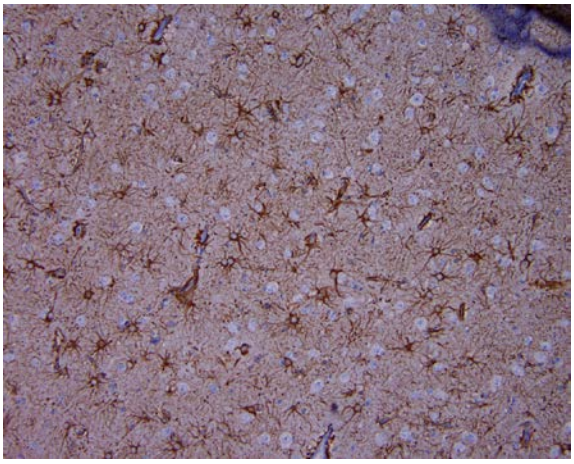


Fig. 18.5 Reactive astrocytes in white matter, stained by anti-GFAP IHC technique with a hematoxylin nuclear counterstain. Fine GFAP-containing processes form a felt-like mat in which the stellate cell bodies are evident.

in the cerebellum are characterized by Bergmann or radial astroglia which have processes running radially from the Purkinje cell layer of the cortex to the pial surface.

In response to injury of the brain parenchyma, astrocytes react by increasing in size with a more prominent, eosinophilic cytoplasm. The nucleus moves from a central to a more eccentric position within the cell cytoplasm and processes become more prominent. Astrocytic gliosis is a response to permanent injury, whereby astrocytes proliferate to fill tissue defects with a fibrous glial scar.

In neuro-oncology, astrocytic differentiation is best demonstrated by GFAP IHC. GFAP immunoreactivity is also seen in other tumors including ependymoma, oligodendroglial tumors and choroid plexus tumors (Eng & Rubinstein, 1978; Velasco et al., 1980; Eng, 1983; Doglioni et al., 1987). Astrocytic tumors also label with vimentin and S-100, but these are also seen in many other tumor types, rendering them of little use for differential diagnoses. Astrocytes occasionally show cross-reactivity as seen for the pan cytokeratin AE1/AE3 (Cosgrove et al., 1989); it is expedient to use other cytokeratin markers such as CAM5.2 or MNF116 to exclude the diagnosis of epithelial tumors.

The proliferation marker Ki-67 (MIB-1) is often used as an aid to grading tumors and to help differentiate reactive from neoplastic astrocytic populations. The latter will tend to have a higher number of nuclei labeled with this marker.

A recently introduced tool for the diagnosis of diffuse oligodendroglial and astrocytic neoplasms is the use of antibodies to isocitrate dehydrogenase 1 (IDH1) carrying the R132H mutation. Approximately 74% of mid-grade (WHO grade II and III) diffuse astrocytomas and oligodendrogliomas have mutations of either *IDH1* or *IDH2*. Tumours with IDH mutations have a better prognosis than those without. The R132H mutation in IDH1 accounts for 90% of such mutations (Hartmann et al., 2009). Commercially available antisera to this mutant protein may be used to differentiate astrocytomas and oligodendrogliomas from their mimics as well as to provide some prognostic prediction (Camelo-Piragua et al., 2010; Capper et al., 2011; Capper et al., 2010).

Oligodendrocytes

These glial cells form the myelin of the central nervous system and are present in both gray and white matter. As noted above, a single oligodendrocyte may myelinate axons from multiple neurons. In H&E and cresyl violet preparations, oligodendrocytes have small (7 μm) round to oval nuclei with compact chromatin. The cytoplasm is indistinct from the surrounding neuropil, although oligodendroglial tumors may show artifactual perinuclear halos in paraffin wax sections. Myelin epitopes for which antisera are commercially available are not expressed by oligodendroglial tumors (Nakagawa et al., 1986). Olig2 is a transcription factor which regulates oligodendroglial development and is expressed by the nuclei of oligodendrocytes and oligodendroglial tumors (Yokoo et al., 2004). Sadly, it is not specific for oligodendrogliomas as it labels other morphologically similar tumors (Preusser et al., 2007) and is also expressed by astrocytomas (Ligon et al., 2004). The authors also find it difficult to use in tissue which has been processed to paraffin wax.

Finally, deletion of chromosomes 1p and 19q (most commonly investigated by fluorescence in situ hybridization) is a well-recognized molecular feature of oligodendrogliomas and appears to be associated with a better prognosis and response to treatment. In the most recent WHO classification of CNS tumors, this co-deletion is considered the defining feature of an oligodendroglioma irrespective of the histomorphological appearance (Louis et al., 2016).

Microglia

These are believed to originate from blood-derived monocytes which move into the brain during embryonic development. They serve as the resident innate immune system and, under certain pathological conditions, may develop into full-blown macrophages. They are involved in most, if not all, known forms of CNS pathology and have a multitude of different behaviors which vary within the pathological and physiological context (Boche et al., 2013). Microglia are subclassified morpholigcally into resting, activated and 'amoeboid' forms. Resting microglia are classically ramified in morphology, whilst activated microglia are rod-shaped and amoeboid are (as the name suggests) amoeboid.

A number of established IHC markers for microglia are available, including CD68 (PGM1), human alveolar macrophage 56 (HAM-56), class II major histocompatibility complex (MHC; particularly in inflammatory states) and HLA-DR-II antibodies. Although these do not label other glial cells, they do label infiltrating macrophages from the circulation.

Neurodegeneration

Neurodegenerative conditions are largely diseases of old age. As the population ages, these conditions place an increasing burden on health and social care systems. This, together with the escalation in research into neurodegeneration in recent years, has resulted in an increasing workload on neuropathology units.

It is often the case that a definitive diagnosis of any neurodegenerative condition cannot be made until autopsy. In most studies, the accuracy of clinical diagnosis of the cause of a dementing illness is in the order of 75%. A neuropathological autopsy does not benefit the deceased, but does have wider benefits, namely:

- It can yield data and tissue to assist research.
- It provides epidemiological data, allowing the prevalence of different neurodegenerative diseases to be monitored.
- Autopsy findings are often of considerable educational benefit for both senior and junior clinicians as well as pathologists.
- An increasing number of neurodegenerative conditions are familial, often with known causative mutations. Accurate neuropathological characterization can therefore guide genetic counseling.

The pathological characterization of neurodegenerative disease is a staged process. The first step is removal of the brain, sometimes with the spinal cord. Following formalin fixation, the brain is examined macroscopically both intact and after slicing (usually coronally). Appropriate blocks of tissue are taken, processed and paraffin wax embedded for microscopy.

In the majority of cases, neuropathological assessment of neurodegeneration tends to broadly focus on dementing illnesses and motor degeneration, with considerable overlap between the two. Investigations of dementia commonly uncover one, or a combination of, three types of pathology, namely: Alzheimer's disease, vascular dementia or dementia with Lewy bodies. A small number of dementia cases show frontotemporal lobar degeneration (FTLD). The neurodegenerative diseases of the motor system which are diagnosed at autopsy tend to focus on motor neuron disease (also known as amyotrophic lateral sclerosis) and conditions which cause Parkinsonian clinical features. Other neurodegenerative diseases of the motor system e.g. Huntington's disease, spinocerebellar ataxia and Friedreich's ataxia tend to be diagnosed by genetic tests.

The microscopic examination of the brain for neurodegeneration is often an iterative process, whereby

Table 18.1 Inclusion body immunostaining

Inclusion	Disease	Immunohistochemistry
Neurofibrillary tangle (Fig. 18.6a, b)	Alzheimer's disease	Tau protein, ubiquitin/p62
Lewy body (Fig. 18.7a)	Parkinson's disease	α-Synuclein, ubiquitin/p62
Cortical Lewy body (Fig. 18.7b)	Dementia with Lewy bodies	α-Synuclein, ubiquitin/p62
Motor neuron disease/FTLD inclusion (Fig. 18.8)	Motor neuron disease, some forms Some forms of FTLD	Ubiquitin/p62, TDP-43
Glial cytoplasmic inclusion	Multiple system atrophy	α-Synuclein, ubiquitin, p62
Glial cytoplasmic inclusion	Progressive supranuclear palsy Corticobasal degeneration	Tau, ubiquitin/p62
Pick body	Pick's disease	Tau protein, ubiquitin/p62

an initial examination is performed using fairly standard preparations, most favoring H&E. After this, more specialist preparations, usually IHC, are performed (Lowe, 1998).

Many neurodegenerative diseases are characterized by accumulations (or inclusions) of protein, and for the majority there are now commercially available antisera. These diseases are often classified by the particular protein which forms the pathological aggregates characterizing the disorders and these inclusion bodies are detailed in Table 18.1. These categories are:

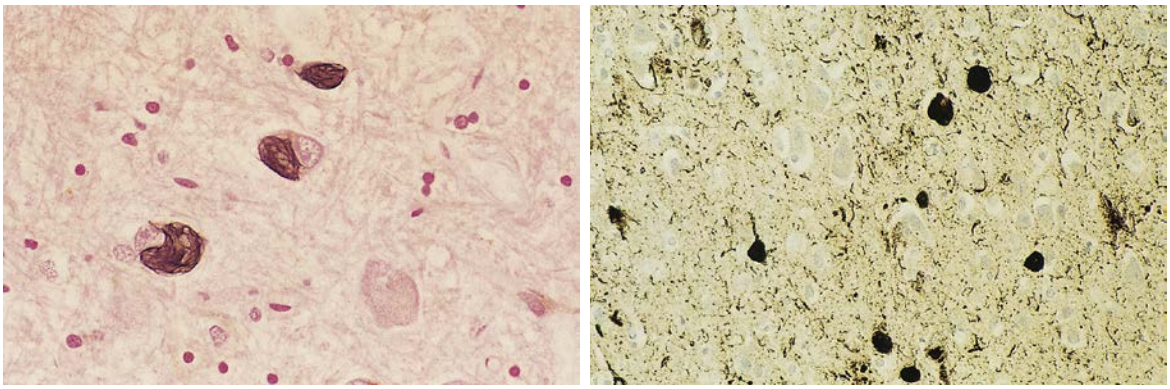
- The *tauopathies*, e.g. Alzheimer's disease, Pick's disease, supranuclear palsy, corticobasal degeneration and argyrophilic grain disease.
- The *synucleinopathies*, Parkinson's disease, dementia with Lewy bodies and multiple system atrophy.
- *Prion disorders* and *TDP-43 proteinopathies*, motor neuron disease, frontotemporal lobar degeneration with TDP-43.

Ubiquitin is a small regulatory protein which binds misfolded or other aberrant proteins, and labels them for destruction by the proteasome. Many dementing illnesses are characterized by accumulations of ubiquitylated protein; the location and form of these accumulations can provide valuable diagnostic clues. Therefore, IHC for ubiquitylated proteins is often a useful early step in neuropathological diagnosis and we favor antibodies to p62. This protein binds ubiquitylated proteins and shuttles them to the proteasome (Wooten et al., 2006). Antibodies to p62 can be used to label pathological, ubiquitylated aggregates of tau, α-synuclein and TDP-43 (Kuusisto et al., 2008). Further, p62 IHC has greater specificity for pathological aggregates than ubiquitin IHC, leaving non-pathological features unlabeled.

As noted above, autopsies in neurodegeneration principally concern dementia and motor system degenerative diseases, many of which overlap. Of the dementing illnesses, the vast majority are diagnosed as Alzheimer's disease, vascular dementia, dementia with Lewy bodies and FTLD. However, given the considerable public health concerns surrounding prion diseases, cases will also occasionally be assessed for this diagnosis.

Alzheimer's disease is characterized by ubiquitylated accumulations of tau and β-amyloid. Hyperphosphorylated tau forms flame-shaped neuronal intracytoplasmic inclusions known as neurofibrillary tangles, and neuritic fibrillary deposits known as neuropil threads. β-Amyloid is formed from aggregates of peptides generated by the cleavage of amyloid precursor protein (a membrane-associated protein of unknown function) by β- and γ-secretases. Deposits of β-amyloid become surrounded by dilated and distorted neuronal processes to form senile plaques. Senile plaques and neurofibrillary tangles are the histological hallmarks of Alzheimer's disease (Figs. 18.6 and 18.9).

Vascular dementia is an umbrella term for a variety of conditions characterized either by multiple



a b

Fig. 18.6 (**a**) Tangles in neurons stained by the Gallyas silver technique. (**b**) Tangles in neurons stained by IHC for phosphorylated tau (AT8). In the background large numbers of neuropil threads can be seen.

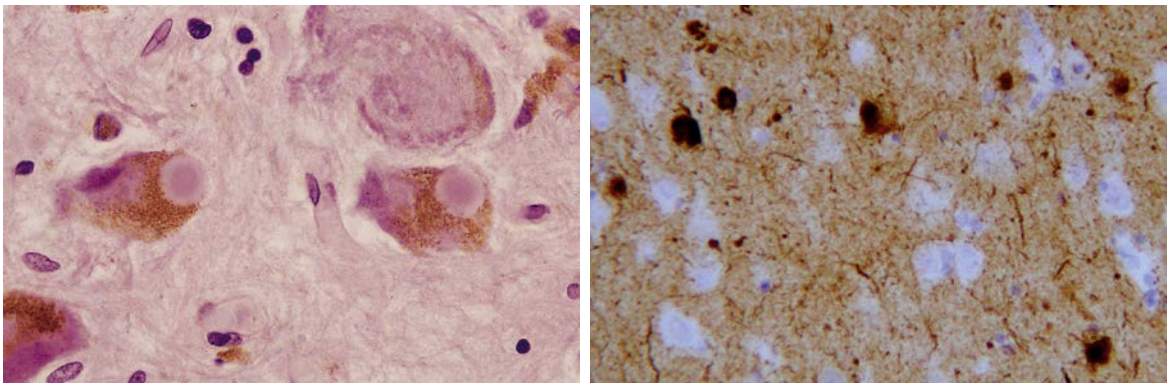
large or small regions of infarction throughout the brain, or by smaller numbers of infarcts in functionally important structures such as the thalamus or hippocampus (Jellinger, 2008). The picture is somewhat complicated by the fact that many cases with a heavy burden of vascular pathology will additionally have a coexistent burden of Alzheimer or Lewy body-type pathology.

Dementia with Lewy bodies is characterized by neocortical and limbic neuronal cytoplasmic inclusions of α-synuclein i.e. the Lewy bodies which give this condition the name. Lewy bodies were first described in Parkinson's disease, where they are visible as round, intensely eosinophilic, hyaline intracytoplasmic neuronal inclusions in the substantia nigra and locus ceruleus of the midbrain and brainstem. They are composed of ubiquitylated α-synuclein and can therefore be detected by IHC for antisera to these proteins or p62 (Dickson, 1999; Goedert, 1999; Kuusisto et al., 2008; Lowe et al., 1993).

Frontotemporal lobar degeneration (FTLD) is an umbrella term for a number of neuropathological entities, all of which predominantly manifest clinically as frontotemporal dementia. The different forms of FTLD are classified by the immunoreactivity of their characteristic proteinaceous intracytoplasmic inclusions (Mackenzie et al., 2010). However, this is a complex and constantly evolving field and classifications tend to rapidly become outdated in their finer details. In essence, the majority of cases are classified as tauopathies due to their accumulations of intracytoplasmic tau. Of the non-tauopathies, most cases show intracytoplasmic inclusions of ubiquitylated, phosphorylated TDP-43 (designated FTLD-TDP), and a few are characterized by inclusions of FUS/TLS or neurofilament. A small residuum is characterized by intracytoplasmic inclusions with immunoreactivity to p62 and ubiquitin alone (FTLD-UPS), or no observable reactivity at all, so-called 'dementia lacking distinctive histology'.

The prion diseases are rare neurodegenerative disorders. They are of considerable interest due to the inherited nature of some forms; the tragic and rapid progression of these conditions; the public health monitoring necessary because of their transmissible nature, and similarly the risk to laboratory staff.

The normal function of prion protein is unclear, and yet it is a normal constituent of cell membranes and most highly expressed in neurons. When misfolded, it is capable of causing disease. In humans, most cases are sporadic, largely Creutzfeldt-Jakob disease (CJD); approximately 10% of cases are familial, and a few have been caused by medical intervention. Prion disease due to the consumption of matter containing misfolded prion proteins is implicated in variant CJD (vCJD) and (historically) kuru (Johnson, 2005).



a b

Fig. 18.7 (**a**) H&E staining of substantia nigra from a patient with Parkinson's disease. The brown color is normal neuromelanin. Two neurons contain Lewy bodies, rounded eosinophilic inclusions with a pale 'halo' around them. (**b**) Immunohistochemistry for α-synuclein showing Lewy bodies and Lewy neurites in the amygdala of a case of dementia with Lewy bodies.

Clinically, sporadic CJD is characterized by a rapidly progressive multifocal neurological dysfunction, sleep disturbance, myoclonic jerks, ataxia and a terminal severe globalized cognitive impairment. Death occurs after approximately 8 months (Johnson, 2005).

Pathologically, CJD is characterized by neuronal loss, gliosis, spongiform change (giving them their alternative name of spongiform encephalopathies), and the deposition of protease-resistant prion protein. From a practical perspective, it should be noted that prion protein is a normal constituent of the nervous system. The pathological form is detected by first treating sections with a protease enzyme to eradicate immunoreactivity to normally folded, physiological prion protein. This leaves immunoreactivity only for pathological, misfolded, protease-resistant prion protein.

Parkinsonism is characterized by tremor, hypokinesia, rigidity and postural instability. It is most commonly caused by Parkinson's disease, which is pathologically characterized by Lewy bodies in the brainstem and midbrain structures (see above and Fig. 18.7). Less common causes include progressive supranuclear palsy and corticobasal degeneration, which are both characterized by neuronal intracytoplasmic accumulations of hyperphosphorylated tau, and multiple system atrophy, which is characterized by glial cytoplasmic inclusions of α-synuclein.

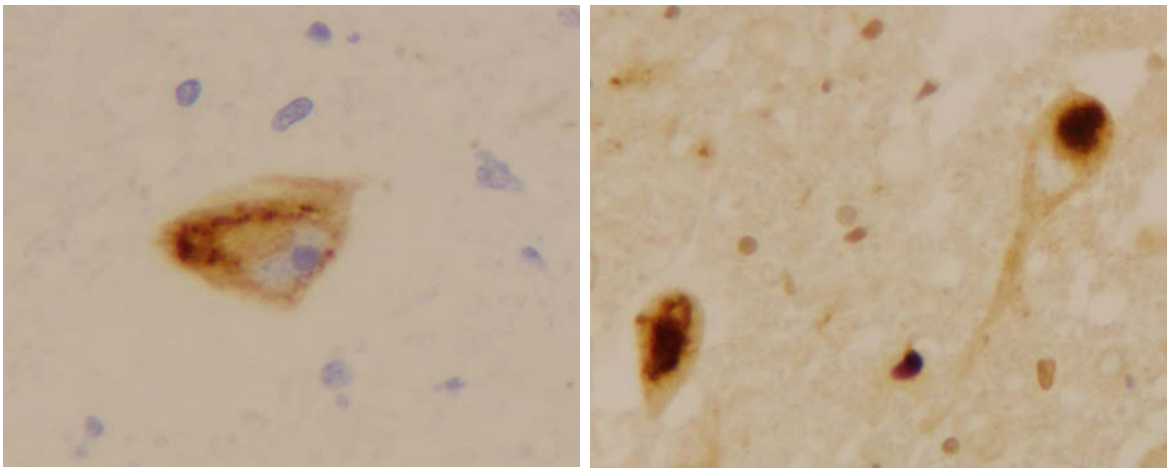
Most cases of **motor neuron disease**, in common with FTLD-TDP, are characterized by neuronal and glial cytoplasmic accumulations of hyperphosphorylated, ubiquitylated TDP-43 (Fig. 18.8).

Following consideration of the principal forms of dementia as described above, it will be apparent that in order to provide a full diagnostic service, a laboratory should have access to optimized IHC for hyperphosphorylated tau, β-amyloid, α-synuclein, p62 or ubiquitin (ideally the former), TDP-43, neurofilament and protease-resistant prion protein.

Stains for detection of the changes of Alzheimer's disease

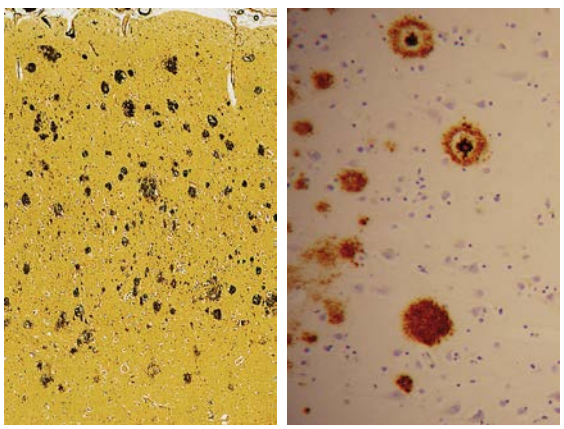
As noted above, there are two types of Alzheimer-type pathology. Firstly, features associated with hyperphosphorylated tau: neurofibrillary tangles and neuropil threads; and secondly, features associated with β-amyloid: neuritic plaques and congophilic amyloid angiopathy. The stains and IHC preparations used in the characterization of Alzheimer-type pathology reflect this dichotomy:

- Silver techniques (e.g. Cross, 1982; Gallyas, 1971) have been replaced by IHC for hyperphosphorylated tau (Fig. 18.6b) in many, but not all centers.



a b

Fig. 18.8 Motor neuron disease (amyotrophic lateral sclerosis). (**a**) p62 IHC showing a skein-like cytoplasmic inclusion in a lower motor neuron. (**b**) TDP-43 IHC showing two motor neurons with 'compact' cytoplasmic inclusions.



a b

Fig. 18.9 Methenamine silver (**a**) and IHC for β-amyloid (**b**), showing senile plaques in cerebral cortex from cases of Alzheimer's disease.

- β-amyloid has historically been demonstrated by methenamine silver (which will also stain a minority of tangles; Fig. 18.9a) and thioflavine S. These have been replaced by IHC after formic acid pretreatment (e.g. BA4; Fig. 18.9b).
- Some preparations can be used to detect both forms of pathology, although these tend to be less sensitive than those preparations which focus on one pathology. Thus, the modified Bielschowsky technique underestimates the β-amyloid pathology load (Lamy et al., 1989).

Guidelines for the dissection and staining of specimens in order to accurately characterize Alzheimer-type pathology have been laid down (Mirra et al., 1993; Alafuzoff et al., 2008a, 2008b, 2009).

Gallyas method for tau pathology (Gallyas, 1971)

This method gives excellent staining of neurofibrillary tangles and the neuritic pathology surrounding plaques, although the amyloid component itself is unstained. It may also be used for a number of other neurodegenerative diseases, especially argyrophilic grain disease.

Fixation

Formalin-fixed tissues.

Sections

Paraffin wax-processed sections, 8 μm thick.

Solutions

5% periodic acid

Alkaline silver iodide solution

Sodium hydroxide	40 g
Potassium iodide	100 g
Distilled water	500 ml
1% silver nitrate	35 ml

Dissolve the sodium hydroxide in water, then add the potassium iodide and wait until dissolved. Slowly add the silver nitrate and stir vigorously until clear. Then add distilled water to give a final volume of 1000 ml.

0.5% acetic acid

Stock solution a

Sodium carbonate (anhydrous)	50 g
Distilled water	1000 ml

Stock solution b (dissolve consecutively)

Distilled water	1000 ml
Ammonium nitrate	2 g
Silver nitrate	2 g
Tungstosilicic acid	10 g

Stock solution c (dissolve consecutively)

Distilled water	1000 ml
Ammonium nitrate	2 g
Silver nitrate	2 g
Tungstosilicic acid	10 g
Formaldehyde (conc.)	7.3 ml

Developer working solution

Add 3 volumes of stock solution **b** to 10 volumes of stock solution **a**. Stir and add 7 volumes of stock solution **c**. Stir and wait to clear.

0.1% gold chloride

1% sodium thiosulfate ('hypo')

0.1% nuclear fast red in 2.5% aqueous aluminum sulfate

Method

1. Take sections to distilled water.
2. Place in 5% periodic acid for 5 minutes.
3. Wash twice in distilled water for 5 minutes.
4. Place in alkaline silver iodide solution for 1 minute.
5. Wash in 0.5% acetic acid for 10 minutes.
6. Place in developer working solution (prepare immediately before use) for 5–30 minutes.
7. Wash in 0.5% acetic acid for 3 minutes.
8. Wash in distilled water for 5 minutes.
9. Place in 0.1% gold chloride for 5 minutes.
10. Rinse in distilled water.
11. Place in 1% sodium thiosulfate solution for 5 minutes.
12. Wash in tap water.
13. Counterstain in 0.1% nuclear fast red for 2 minutes.
14. Wash in tap water.
15. Dehydrate, clear and mount in DPX.

Results

Neurofibrillary tangles and plaque neurites	black
Nuclei	red

Methenamine silver method for senile plaques (Yamaguchi et al., 1990)

This preparation has largely been superseded by βA4 IHC. It stains amyloid plaques well, but detects only a subset of neurofibrillary tangles.

Fixation

Formalin fixed.

Sections

Paraffin wax, 8 µm.

Solutions

Working solution

5% hexamine	50 ml
5% sodium tetraborate	2.5 ml
5% silver nitrate	2.5 ml

Add the reagents in the above order, the silver nitrate must be last.

10% formalin in tap water

Method

1. Take sections to water.
2. Rinse in distilled water.
3. Place sections in working solution for 3–4 hours at 60°C.
4. Check microscopic appearance of plaques and tangles at regular intervals until stained black.
5. Rinse in distilled water.
6. Place sections in 10% formalin in tap water for 5 minutes.
7. Rinse in tap water.
8. Place sections in 5% sodium thiosulfate for 5 minutes.
9. Rinse in tap water.
10. Dehydrate, clear and mount in DPX.

Results

Amyloid plaques	black
Some tangles (rare)	black
Background	yellow/brown

Modified Bielschowsky method for plaques and tangles (Litchfield & Nagy, 2001)

This preparation is a reasonable compromise between plaque and tangle labeling. It can therefore be used for diagnostic purposes.

Fixation

Formalin fixed.

Sections

Paraffin wax sections, cut at 6–8 µm.

Solutions

20% silver nitrate solution

Silver nitrate	20 g
Distilled water	100 ml

Developer

Formalin	20 ml
Distilled water	100 ml
Concentrated nitric acid	1 drop
Citric acid	0.5 g

0.2% ammonia washing solution

Ammonia	0.2 ml
Distilled water	100 ml

1% sodium thiosulfate ('hypo')

Method

1. Take sections to water.
2. Place slides in 20% silver nitrate for 20 minutes in a fridge at 4°C.
3. Place slides in distilled water while performing step 4 below.
4. To the 20% silver nitrate (used in step 2) add ammonia (sp. grade 0.880), drop by drop, stirring vigorously until the precipitate turns clear. Add two more drops of ammonia. Return slides to this solution for 15 minutes in a fridge.
5. Place slides in 0.2% ammonia. This is a holding step whilst the working developer solution is made.
6. To 50 ml of the ammoniacal silver solution (from step 4), add 3 drops of the developer solution; place this in a clean Coplin jar. Drain the slides of the ammonia, wash and place in the ammoniacal silver/developer solution. The development time will vary between approximately 2 and 5 minutes, depending on the ambient temperature. As the development proceeds the tissue will turn a golden brown. Check microscopically until the tangles and plaques are optimally demonstrated.
7. Wash in distilled water.
8. Fix sections in 'hypo' for 5 minutes.
9. Wash in distilled water.
10. Dehydrate, clear and mount in DPX.

Results

Tangles	black
Plaques	black
Background	brown

Notes

a. Some laboratories have found that variable ambient room temperature causes variability and inconsistency in staining. Performing the silver and ammoniacal silver stages at 4°C overcomes this problem. If this is not a concern for a particular laboratory, these steps can be performed at room temperature.

b. The original method used separate solutions of 20% silver at steps 1 and 4; however, with meticulous technique it is more cost-effective to use the same silver solution throughout.

Neuropathology laboratory specimen handling

Molecular neuroscience has flourished in recent years and neuropathology laboratories find that they are only one part of a larger team dedicated to the diagnosis and characterization of neurological diseases. The neuropathology service, therefore remains central to the handling and storage of tissue as well as the procurement of that tissue, either by biopsy or autopsy. The department must now do this in a manner which facilitates the work of other disciplines using the same tissue.

The principal histological specimens submitted to or taken by the neuropathology service are:

- Neurosurgical biopsies and excision specimens.
- Peripheral nerve biopsies.
- Muscle biopsies. (See later, and previous editions.)
- Brain, spinal cord and other specimens taken at autopsy.

Brain and spinal cord biopsies and excision specimens

Given the declining autopsy rate which is seen internationally (Burton & Underwood, 2007), surgical samples of tissue from the CNS are coming (or have already come in many countries, including the UK) to represent the bulk of neuropathological practice.

The majority of these specimens are taken for the diagnosis and/or treatment of neoplasia. The tissue samples are often small and require careful handling. Some centers prefer neurosurgical biopsies to be placed on a sterile polythene sheet which is folded over the specimen to reduce drying, contamination or loss. Other centers prefer samples to be placed directly into a clean pot. The biopsies should be transported

rapidly to the neuropathology laboratory. On arrival at the laboratory, different portions of the fresh tissue may be sampled for intraoperative diagnosis by smear; snap frozen for molecular analysis or intraoperative diagnosis with cryostat sections; or fixed in glutaraldehyde for electron microscopy. Following this, the remainder of the specimen is fixed in formalin. If this is not possible, the biopsies should be placed in formalin fixative. Adequate formalin should be used, a minimum of 10 times the volume of the tissue.

Poorly handled tissue is a source of frustration for any neuropathology service. Anyone who has worked in such a laboratory will be well acquainted with the small tube into which a large tumor has been stuffed, which requires a second 'neurosurgical' procedure to extract it, piecemeal, poorly fixed and terribly disrupted. The hopelessly desiccated fragment of tissue stuck to the bottom of a glass tube which has taken several hours to reach the laboratory is also well known. We could go on, but instead beg that histology staff strive to educate their neurosurgical and theater colleagues in the appropriate handling of tissue!

Intraoperative diagnosis from smear preparations and frozen sections

The histological and cytological detail which can be achieved from smear and frozen section preparations is substantially less than can be achieved from paraffin wax based histology. Diagnoses made by these techniques can only be viewed as an approximation and subject to review following paraffin wax histology. As there is often only a small amount of tissue available, it is possible that performing an intraoperative diagnosis may compromise the ability to achieve a definitive diagnosis on paraffin wax-based histology. A feature which is crucial for diagnosis may be lost in the tissue used for smear or frozen section. It is vital that prior to making smear or frozen section preparations from small biopsies the neuropathology staff are certain that this is necessary.

To produce smears, representative small pieces of tissue, approximately 1 mm in diameter, are dissected from the biopsy and placed at one end of a plain glass microscope slide. A second glass slide is used to crush the specimen and it is then drawn across the slide to produce a uniform smear. Unlike blood film preparation, the two slides are held flat together during smearing, maintaining a gentle and even pressure. Alternatively, the tissue may be lightly touched to a single glass slide, allowing a few cells to adhere to the slide in order to minimize handling artifacts. The slides are immediately fixed in acetic alcohol (Wolman's solution) and stained with H&E, aqueous toluidine blue or both. We find that the former gives better cytoplasmic definition and the latter more nuclear detail. All cell types in the CNS are readily identifiable by this method (Moss et al., 1997). Certain lesions may be too tough to smear, in which case a frozen section may be used.

Central nervous system tissues taken at autopsy

After removal and macroscopic examination in the autopsy room, samples may be taken and snap-frozen for studies which require unfixed tissue. The brain should then be immersed in a large bucket of formalin. It should be suspended by the basilar artery from a piece of string tied to the two attachments of the bucket handle. This may be done by passing the string under the basilar artery, or by hooking the basilar artery to the string with a curtain hook or safety pin. The latter method is best avoided as the safety pin tends to rust. The brain should not be allowed to touch the bottom or sides of the bucket as this produces unacceptable distortion and it should be left to fix for 3 or more weeks. The spinal cord may be suspended by the dura mater in a suitably long measuring cylinder with a weight attached to the dura mater at the lower end, avoiding artifactual contraction of the cord during fixation. Whilst some laboratories prefer the minimization of tissue distortion which this method provides, others consider this is an unnecessarily complicated method and prefer to immerse the cord in the brain bucket beneath the fixing brain.

Whilst paraffin wax processing of neurosurgical material can be carried out using most routine overnight schedules, autopsy CNS material requires longer processing, depending upon block size. This can take anything from 48 hours to 5 days. Central nervous system tissue has a high lipid content due to the presence of myelin. This

lipid makes it prone to processing artifact, largely due to poor dehydration. For this reason, some laboratories use isopropyl alcohol for the last two changes of alcohol to improve tissue dehydration during tissue processing.

Processing artifacts include:

- Sections blowing apart on the water bath.
- Tissue 'crazing', whereby sections start to disintegrate, producing 'crazy paving'-like cracking, usually at the edge of the section.
- Sections lifting off the slide, especially in the context of IHC on jumbo slides.
- Poor staining.

Most of these problems can be reduced by using a longer processing schedule and the use of coated/charged slides. Rushing fixation, processing or drying slides will lead to suboptimal preparations and frustration.

The prion diseases present special methodological problems for autopsy and subsequent tissue handling. The misfolded prion, being the transmissible element of these diseases, is a Group 3 Hazard and is extremely resistant to usual decontamination methods. Dissection of a brain where prion disease is a possibility should be done in an appropriate laboratory containment facility using disposable instruments and appropriate protective clothing. All contaminated tissues and fluids should be contained. Sampled tissues should be treated with formic acid prior to paraffin wax embedding (Brown et al., 1990). Work surfaces and instruments may be sterilized in 2M sodium hydroxide for 1 hour. Glassware can be cleaned in sodium hypochlorite (20,000 ppm). In cases of vCJD, prion protein (an infective hazard) also resides in lymphoid tissues such as tonsil, lymph node, spleen and bone marrow (Ironside et al., 2000). Detailed protocols for the handling of tissues which may be affected by prion disease have been published (Bell & Ironside, 1993; Department of Health, 2011; World Health Organization, 2004).

Peripheral nerve biopsies

These provide clinically useful information in the management of infection, inflammatory and immune disorders e.g. vasculitis and granulomatous diseases, amyloidosis and neoplasia (Dyck et al., 2005).

Peripheral nerve tissue is exceptionally delicate and prone to artifacts due to handling, and it is imperative that this is kept to a minimum. In this context it is worth recalling that myelin is a liquid, such that pressure on the nerve at one point will result in pressure fluctuations which cause disruption at locations away from the site of compression. The fragility of nerve biopsies make it imperative that they should be performed only by specialist staff, usually a neurosurgeon or neurologist, who are trained and experienced in this procedure. The number of practitioners should be minimized so as to concentrate the expertise in these individuals. Useful technical guidelines for the taking, processing and interpretation of nerve biopsies have been published (Sommer et al., 2010).

Peripheral nerve biopsy should only be performed after consultation between the laboratory and clinical teams. A minimum of 20 mm, and preferably 30 mm, of nerve should be taken. Ideally, a member of the neuropathology technical staff should attend the biopsy room in order to receive the specimen as soon as possible. This should be placed gently on a piece of dry card, to which it will naturally adhere. The nerve should not be put in fixative, sutured, clamped, stapled, pinned or traumatized in any way.

On arrival at the laboratory, a fresh scalpel blade is used to divide the biopsy into at least three segments: one for paraffin wax based histology, one for semi-thin preparations and electron microscopy, and one for teased fiber preparations. Further portions may be taken for snap freezing. The 2 mm portions at the ends of the biopsy are usually damaged at surgical removal and are thus inappropriate for histology. They may, however, be frozen for molecular studies. Glutaraldehyde is the preferred fixative for processing to semi-thin sections and electron microscopy. Opinion is divided on the best fixative for paraffin wax based nerve histology, although many prefer formalin (10% NBF).

Following glutaraldehyde fixation, the nerve may be cut into 1 mm pieces, osmicated if necessary, processed and plastic embedded (see also Chapters

8 and 21). These blocks may be used for electron microscopy or the preparation of semi-thin (1–3 μm) sections. Semi-thin sections of transversely oriented tissue are cut and stained with toluidine blue or methylene blue-azure II-basic fuchsin.

Paraffin wax processed material is cut at 6–8 μm. It is helpful to include a transverse and a longitudinal segment for examination. Different centers vary in the stains prepared as a matter of routine. These may include H&E, a trichrome stain to assess fibrosis, a myelin stain, an axon stain and an amyloid preparation such as Congo red. Many biopsies are performed for inflammatory disorders, e.g. vasculitis. As these can be focal, patchy disorders, serial sections through the paraffin wax block should be performed and it may be necessary to augment this with IHC.

Methylene blue azure II-basic fuchsin (based on Humphrey & Pittman, 1974)

Fixation

Glutaraldehyde.

Sections

Semi-thin resin sections.

Solutions

0.1 M phosphate buffer pH 6.9

Methylene blue/azure A

Methylene blue	0.39 g
Azure A	0.06 g
Glycerol	30 ml
Methanol	30 ml
0.1 M phosphate buffer, pH 6.9	90 ml
Distilled water	150 ml

50% ethanol in deionized water

Basic fuchsin, stock solution

Basic fuchsin	0.5 g
50% ethanol in deionized water	50 ml

Basic fuchsin, working solution

Basic fuchsin stock solution	1 ml
Deionized water	19 ml

Method

1. Filter methylene blue/azure A solution into a Coplin jar.
2. Put into a water bath set at 65°C.
3. Stain sections in this solution for 30 minutes.
4. Rinse well in distilled, filtered water.
5. Do not allow sections to dry.
6. Filter basic fuchsin working solution onto slides and stain at room temperature for 4 minutes.
7. Rinse well in distilled water.
8. Drain, and allow to dry.
9. Mount in DPX.

Result

Myelin	blue
Other tissue elements	light blue
Collagen	pink/red
Elastin	red

Notes

a. If staining for leprosy bacilli in sections, stain in filtered working solution of basic fuchsin for a strict 2 minutes only. Follow this by washing in distilled water.

b. Do not leave sections stained with methylene blue/azure A in distilled water before counterstaining as the blue will wash out and result will be too pale.

Teased fiber preparations allow the examination of the pattern of myelin formation along individual axons (Asbury & Johnson, 1978). This can give information on whether there have been past episodes of myelin loss with re-myelination, or whether there is myelin fragmentation due to axonal loss.

Preparation of teased nerve fibers (Asbury & Johnson, 1978)

Nerve fibers can be processed to glycerol or unpolymerized Araldite for teasing. The latter method produces a firmer consistency to the individual fibers and is easier to work with. The stiffness of the fibers also relates to the amount of osmication and the concentration of the osmium used.

Tissue

Fresh nerve.

Fixation

Segment of nerve is fixed in 0.1 M phosphate-buffered 3.6% glutaraldehyde, for 4–16 hours.

Method

1. Wash twice in phosphate buffer for 15 minutes.
2. Under a stereomicroscope using two pairs of fine forceps, carefully remove the epineurium by only gripping and pulling the connective tissue. Separate the nerve into individual or small bundles of fascicles. This allows a uniform osmication, disregarding the variation in size of the specimen which one may have received.
3. Osmicate in 0.1 M phosphate-buffered 2% osmium tetroxide for 4 hours.
4. Wash twice in phosphate buffer for 15 minutes each.
5. Briefly rinse in distilled water and process through 50, 80, and 95% alcohol for 10 minutes each.
6. Dehydrate in two changes of 100% alcohol for 15 minutes each.
7. Process through two changes of propylene oxide, 15 minutes each.
8. Mix in equal parts of propylene oxide and Araldite CY212 resin for 1 hour.
9. Mix in unpolymerized Araldite CY212 resin overnight (the specimen can be kept in this resin at 4°C for up to 1 year).

Notes

- **a.** Laboratories which do not have access to Araldite processing can use a simplified method where, following fixation, the nerve biopsy is washed in distilled water (1 hour), osmicated (4 hours to overnight), washed in distilled water (1 hour), treated with 60% glycerol overnight, and then teased as described below.
- **b.** Any remaining tissue from the protocol outlined in note a can be subsequently paraffin wax processed and stained with H&E.

To tease fibers after preparation, place the processed nerve on a glass slide under a stereo-microscope in a pool of unpolymerized Araldite. Using fine forceps and sharp needles, remove the perineurium from a fascicle. Keep dividing the nerve bundles into halves until single or small bundles of two to three fibers (black) can be carefully teased out. When separating a smaller bundle from a larger one, it is helpful to hold onto the smaller one and pull the larger one slowly away. Slide the fiber across the slide in a trail of resin onto an adjacent slide. Care should be taken to ensure that the fibers to be examined are aligned in parallel across the slide.

For diagnostic purposes, in order to avoid possible sampling errors, at least 100 fibers should be sampled (Dyck et al., 2005). In severely demyelinated cases, it may prove difficult to get enough black fibers. When enough fibers are obtained, use the fine forceps to pick up a small droplet of partially polymerized resin. Apply a thin line of the resin along one of the aligned ends of the parallel-arranged fibers, the resin should not touch the fibers at this stage. Hold the coverslip at an angle to the surface of the slide and carefully touch the line of resin with the lower edge of the coverslip. Carefully lower the coverslip until it almost touches the slide, and let go. Lay the slide on a flat surface in a 37°C oven and let the resin slowly spread longitudinally along the fibers to fill the entire gap between the slide and the coverslip. Ring the coverslip with nail varnish. Any trapped bubbles should be left undisturbed. With practice, this mounting technique will allow the well-aligned, loosely attached fibers to remain undisturbed and without overlapping on the slide.

Muscle biopsies

Biopsy samples of skeletal muscle may be taken either using a biopsy needle or at open operation. The aim of histology is to provide an undistorted picture of muscle fiber architecture. In contrast to many areas of histopathology, cryostat sections of unfixed skeletal muscle are the mainstay of diagnostic practice as they offer a better visualization of structure and pathology, as well as allowing enzyme histochemistry to be performed (Table 18.2).

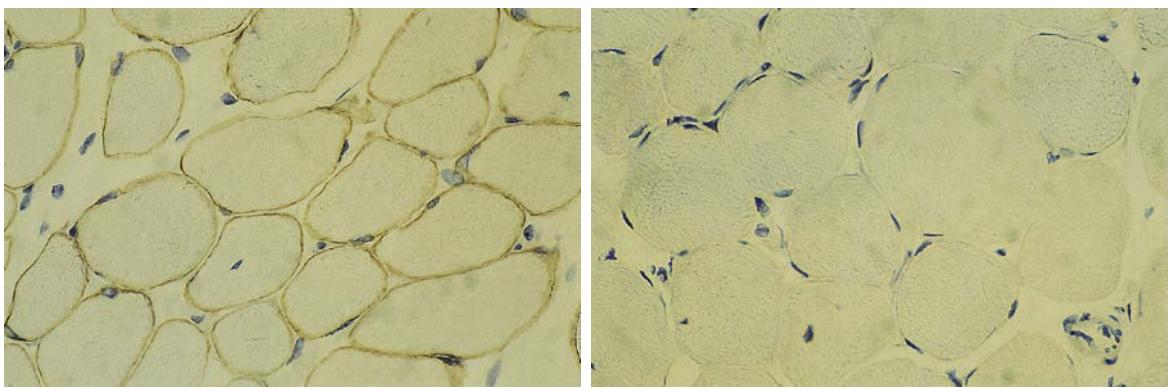
A small portion of muscle may be fixed for electron microscopy, if clinically indicated. The sample may be fixed lightly stretched longitudinally, in a special clip, or stretched out by pinning and fixed with a few drops of buffered glutaraldehyde. After a few minutes, it will be stiffened and can be put into the main bulk of fixative.

Major histocompatibility complex (MHC) class I proteins are overexpressed in some inflammatory myopathies, a feature which can be detected by IHC.

Table 18.2 Staining methods for muscle biopsies

Method	Demonstrates
H&E	Morphology
Gomori trichrome	Inclusion bodies, connective tissue, ragged red fibers and tubular aggregates
PAS ± diastase	Glycogen
Oil Red O	Lipids
ATPase[1], pH 9.4	Myosin loss and myofiber atrophy of Type 1 and 2 fibers
ATPase, pH 4.6	Type 2B myofibers
ATPase, pH 4.3	Type 2C myofibers
NADH-TR[2]	Internal fiber architecture, mitochondria and tubular aggregates
Alkaline phosphatase	Regenerating myofibers, autoimmune connective tissue disorders
Acid phosphatase	Inflammatory cells, necrotic fibers, enhanced lysosomal enzyme activity
Non-specific esterase	Inflammatory cells, necrotic myofibers, enhanced lysosomal enzyme activity
Cytochrome c oxidase	Mitochondrial disorders
Succinic dehydrogenase	Mitochondrial disorders
Myoadenylate deaminase	Enzyme deficiency
Myophosphorylase	Type V glycogenosis (McArdle's disease)
Dystrophy-related IHC stains	Dystrophin 1, 2, and 3 (Fig. 18.10a & b), sarcoglycans (α, β, γ, and δ), dysferlin, merosin, caveolin, emerin, calpain-3, spectrin

[1]ATPase, adenosine triphosphatase.
[2]NADH-TR, nicotinamide adenine dinucleotide dehydrogenase reductase.



a b

Fig. 18.10 (**a**) In a normal muscle, dystrophin is localized beneath the cell membrane of muscle fibers. (**b**) In Duchenne muscular dystrophy, this staining pattern is absent.

A detailed description of muscle histology and biopsy handling is given by Dubowitz and colleagues (Dubowitz et al., 2013).

The Gomori technique and its modifications allows the identification of abnormal mitochondrial aggregates, the rimmed vacuoles of inclusion body disorders and 'ragged red' fibers suggestive of mitochondrial cytopathies.

Glycogenoses tend to result in the accumulation of glycogen, which can be demonstrated by

the periodic acid-Schiff reaction (PAS; see page 183), whilst carnitine deficiency may result in the accumulation of lipid which may be demonstrable by the Oil Red O or Sudan Black reactions (see following).

Modified Gomori trichrome (Engel & Cunningham, 1963)

Sections

Unfixed cryostat.

Solutions

Gomori trichrome solution

Fast Green FCF	0.3g
Chromotrope 2R	1.2g
Phosphotungstic acid	0.6g
Glacial acetic acid	1 ml
Distilled water	100 ml

Adjust the pH to 3.4 with 1 M NaOH.

Method

1. Stain sections with filtered Harris' hematoxylin for 5 minutes.
2. Wash sections well with distilled water. (Do not use tap water, the nuclei must remain red not blue).
3. Stain sections with filtered Gomori trichrome stain for 15 minutes.
4. Wash section well with distilled water.
5. Rinse briefly in 0.5% acetic acid, and then in distilled water. This helps differentiate excess chromotrope 2R out of the muscle fibers, and also it helps to retain the red coloration to the nuclei.
6. Dehydrate, clear and mount sections in DPX.

Result

Nuclei & mitochondria	red
Muscle fibers	green/blue
Nemaline rods	red
Membranous whorls of rimmed vacuoles	red
Myelin of nerves	red
Connective tissue	pale green

Notes

Good quality dyes should only be used to make up this trichrome solution. If the staining becomes pale, replace the solution.

Oil Red O stain for neutral lipid (Lillie & Ashburn, 1943)

Sections

Unfixed cryostat.

Oil Red O stock solution (ORO)

Oil Red O	1g
60% Triethyl phosphate	100 ml

Working solution of ORO

Dilute 6 ml of stock ORO with 4 ml of distilled water. Stand for 10 minutes and filter solution.

Method

1. Treat sections in ORO for 10-30 minutes.
2. If the sections are overstained, they can be rinsed in 60% isopropyl alcohol to clean the background up.
3. Rinse in distilled water.
4. Counterstain in Harris' hematoxylin for 1 minute.
5. Wash in tap water to blue up.
6. Mount in an aqueous mountant.

Results

Neutral lipid	red
Nuclei	blue

Type 1 muscle fibers contain more lipid droplets than type 2 fibers.

Notes

a. Isopropyl alcohol can be used instead of triethyl phosphate.
b. To help the dye dissolve more rapidly in triethyl phosphate, the solution can be warmed at 56°C for 2-3 hours.

Sudan Black B

Sudan Black B is a fat-soluble diazo dye which stains neutral triglycerides and lipids. It can be adapted to mask the autofluorescence of lipofuscin during immunofluorescence procedures. Many centers find it superior to Oil Red O as the latter tends to result in lipid droplets being lost from the tissue and becoming deposited at random places on the microscope slide. Sudan Black is less prone to this artifact.

Sections

Unfixed cryostat, 7-9 µm thick.

Solution

70% industrial methylated spirit	200 ml
Sudan Black B powder	Approximately 3 g

Add the Sudan Black B until the powder no longer dissolves and is seen in the base of the container. Mix well. This stock solution can be stored at room temperature for approximately 2 months.

Method

1. Rinse sections in 70% alcohol.
2. Filter Sudan Black B solution twice.
3. Place slides to stain in the filtered Sudan Black B in a slide holder for 30 minutes.

4. Rinse the slide in tap water for 5 minutes.
5. Stain in a Gill's hematoxylin for 2 minutes.
6. Rinse the slide in tap water for 10 minutes.
7. Mount in aqueous mounting medium.

Results

The Sudan Black B technique should demonstrate a fine blue/black granular-like appearance of lipid scattered within normal muscle fibers. Nuclei are blue.

Enzyme histochemistry

These techniques serve a number of purposes in muscle. Firstly, the ATPase and NADH-TR reactions allow an appreciation of the specific muscle types and thus highlight the selective involvement of fiber types in pathological processes. Secondly, they can highlight aspects of the myofiber architecture which cannot be seen on tinctorial preparations. Finally, these may show an absence of a given enzyme activity, e.g. phosphorylase in type V glycogenosis.

Adenosine triphosphatase, ATPase (Round et al., 1980)

This procedure allows different fiber types to be identified. Some laboratories are dropping this in favor of IHC for different myosin subtypes, but many still prefer these methods.

Sections

Unfixed cryostat.

Solutions

0.1M glycine buffer

Glycine	0.75g
NaCl	0.585g

Make up to 100 ml with distilled water.

0.1M glycine buffer with 0.75M $CaCl_2$

0.1M glycine buffer	50 ml
0.75M $CaCl_2$ (11.03g·$CaCl_2$·$2H_2O$ in 100 ml distilled water)	10 ml

Mix, then add approximately 22 ml 0.1M NaOH until pH 9.4.

0.1M solution sodium acetate buffer with 10 mM EDTA pH 4.3 and pH 4.6

Incubating solution

ATP	5 mg
0.1M glycine buffer with 0.75M $CaCl_2$	10 ml

Adjust to pH 9.4 with 0.1M NaOH or 0.1M HCl if necessary.

Method (at pH 9.4)

1. Incubate sections in incubating solution for 30 minutes at 37°C.
2. Rinse well in distilled water.
3. Immerse in 2% cobalt chloride for 5 min.
4. Rinse well in tap water, then in three changes of distilled water.
5. Immerse in dilute (1:10) ammonium sulfide solution for 30 seconds (in a fume cupboard).
6. Rinse well in running tap water.
7. Stain lightly in Harris' hematoxylin, blue in tap water (optional step).
8. Mount in aqueous mountant or dehydrate, clear and mount in DPX.

Method (at pH 4.3 and 4.6)

1. Pre-incubate freshly cut sections at 4°C in appropriate 0.1M sodium acetate buffer for 10 min.
2. Rinse briefly in distilled water.
3. Proceed as from step 1 in the pH 9.4 method above.

Results

Checkerboard pattern of black and white fibers:
pH 9.4 – type 1 white, type 2 black (type 2A may show an intermediate intensity), 2C black.
pH 4.6 – type 1 black, 2A white, 2B intermediate, 2C black.

pH4.3 – type 1 black, 2A and 2B white, 2C black or intermediate.

Note

Sections must be well washed after the cobalt chloride and ammonium sulfide steps.

Reduced nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase, NADH-TR (Pearse, 1972)

Sections

Unfixed cryostat.

Solutions

Nitro blue tetrazolium (NBT) stock solution

Nitro blue tetrazolium	20 mg
Distilled water	20 ml

Store in aliquots at -20°C.

NADH stock solution

NBT stock solution	6.25 ml
0.2 M Tris buffer pH7.4	1.25 ml
Cobalt chloride 0.5 M	1.25 ml
Distilled water	8.75 ml

Can be stored in aliquots at -20°C.

Incubating solution

NADH stock solution	1 ml
NADH	1 mg

Method

1. Incubate sections in incubating solution at 37˚C for 30 minutes.
2. Drain sections and place directly into 10% formalin in tap water for 15 minutes.
3. Wash sections well in tap water.
4. Dehydrate through graded alcohols to xylene.
5. Mount in DPX.

Results

Blue/grey end product:

Higher activity in type 1 fibers and in areas with mitochondrial aggregates; Type 2B fibers weakest; 2A intermediate intensity.

Note

The formalin step stabilises and darkens the formazan reaction product.

Succinate dehydrogenase, SDH (Kiernan, 1981; Dubowitz et al., 2013)

Sections

Unfixed cryostat.

Solutions

0.2 M phosphate buffer

Solution a

0.2 M Potassium dihydrogen orthophosphate (2.72 g in 100 ml distilled water)

Solution b

0.2 M di-sodium hydrogen orthophosphate (0.57 g in 20 ml distilled water)

Working phosphate buffer

Solution **a**	4 ml
Solution **b**	16 ml

0.2 M sodium succinate

Sodium succinate	1.08 g
Distilled water	20 ml

SDH incubating solution

0.2 M phosphate buffer	15 ml
0.2 M sodium succinate	15 ml
Nitro blue (NBT)	30 mg

Adjust pH to 7.3–7.6. Can be stored in aliquots at -20˚C.

Method

1. Incubate sections in incubating solution at 37˚C for 1–2 hours.
2. Drain sections and place directly into 10% formalin in tap water for 15 minutes.
3. Wash sections well in tap water.
4. Dehydrate through graded alcohols to xylene.
5. Mount in DPX.

Results

Type 1	blue/grey end product (high activity), also areas with mitochondrial aggregates.
Type 2A	pale weak blue/grey end product (intermediate activity).
Type 2B	weak blue/grey end product (low activity).

Notes

a. The formalin step stabilises and darkens the formazan reaction product.
b. This reaction is often combined with the cytochrome oxidase (COX) reaction (below).

Cytochrome oxidase, COX (Seligman et al., 1968)

Sections

Unfixed cryostat.

Incubating solution

Catalase (20 µg/ml) (4 mg in 10 ml; remove 2.5 ml and make up to 50 ml in distilled water)	1 ml
Cytochrome c (type 2)	10 mg
0.1 M phosphate buffer, pH 7.4	9 ml
3,3′-Daminobenzidine tetrahydrochloride (DAB)	5 mg

Adjust pH to 7.4 before use with 0.1M NaOH or 0.1M HCl as required. Can be stored in aliquots at -20˚C.

Method

1. Incubate sections at 37˚C for 2–3 hours.
2. Rinse in distilled water.
3. Fix in formal calcium for 15 min.
4. Wash and blue.
6. Dehydrate, clear and mount in DPX.

Results

Brown reaction product at sites of cytochrome oxidase activity.

Notes

a. All muscle fibers should demonstrate activity, but type 1 fibers are the most strongly positive.
b. All fibers are negative in congenital cytochrome oxidase deficiency.
c. Occasional negative fibers are seen in aging, and in mitochondrial cytopathy syndromes.
d. 10% formalin in tap water can be used for step 3.
e. DAB is carcinogenic: handle with care.

Alkaline phosphatase: azo dye coupling method using α-naphthyl phosphate

Useful for some forms of myositis.

Fixation

Formal calcium at 4°C. Formal vapor.

Sections

Pre-fixed cryostat preferred.

Preparation of incubating medium

Sodium α-naphthyl phosphate	10 mg
0.2M Tris buffer pH 10.0	10 ml
Diazonium salt (fast red TR)	10 mg

The final pH of the incubating medium should be between 9.0 and 9.4. The sodium α-naphthyl phosphate is dissolved in the buffer, the diazonium salt is added and the solution well mixed. The solution is then filtered and used immediately.

Method

1. After fixation, bring sections to water, incubate at room temperature for 10–60 mins.
2. Wash 3 minutes in distilled water.
3. Wash 2 minutes in 1% acetic acid.
4. Rinse in distilled water.
5. Mount in aqueous mountant.

Results

Alkaline phosphatase activity reddish/brown.

Acid phosphatase: the naphthol AS-BI phosphate method (Burstone, 1958; Barka, 1960)

This method is useful for lysosomal storage disorders and vacuolar myopathies.

Fixation

Formal calcium at 4°C. Formal vapor.

Sections

Pre-fixed cryostat preferred (but unfixed works).

Preparation of solutions

Substrate solution a

Naphthol AS-BI phosphate	10 mg
Dimethyl formamide	1 ml

Buffer solution b

Sodium acetate ($3H_2O$)	1.94 g
Sodium barbitone	2.94 g
Distilled water	100 ml

Sodium nitrite solution c

Sodium nitrite	400 mg
Distilled water	10 ml

Pararosaniline HCl stock solution d

Pararosaniline hydrochloride	1g
Distilled water	20 ml
Hydrochloric acid (conc.)	5 ml

Heat gently, cool to room temperature, and filter.

Preparation of incubating solution

Solution **a**	0.5 ml
Solution **b**	2.5 ml
Solution **c**	0.4 ml
Solution **d**	0.4 ml
Distilled water	6 ml

For the success of this technique it is essential that equal parts of solutions **c** and **d** are mixed together first and allowed to stand for 2 min before being added to solutions **a** and **b**. The final pH should be between 4.7 and 5.0, it is adjusted if necessary with 0.1M NaOH.

Method

1. Incubate sections at 37°C for 15–60 min.
2. Wash in distilled water.
3. Counterstain in hematoxylin.
4. Wash in running water.
5. Either mount in aqueous mountant or dehydrate rapidly through fresh alcohols to xylene and mount in DPX.

Results

Acid phosphatase activity	red
Nuclei	blue

Note

This is a reliable method giving sharp localization of the enzyme. There are three points to watch in the preparation of the incubating solution: that solution **c** (sodium nitrite) is fresh, the pH of the final incubating solution is correct, and the incubating solution is filtered.

Myoadenylate deaminase (Fishbein et al., 1978)

Some centers use this to assess exertional myalgia. However, as 1-2% of the population is deficient for this enzyme, the significance of the loss of activity is unclear.

Sections

Unfixed cryostat.

Solution

Adenosine 5′-monophosphate	4 mg
Distilled water	7.0 ml
Nitro blue tetrazolium (5 mg/ml in distilled water)	2.0 ml
3M potassium chloride	0.7 ml

Add potassium chloride slowly while stirring. Adjust pH to 6.1, then add, dropwise, 5 mg dithiothreitol dissolved in 0.3 ml distilled water just before using.

Method

1. Incubate at room temperature for 1 hour.
2. Rinse briefly in distilled water.
3. Mount in glycerin jelly.

Results

The deep blue stippled reaction end product is more intense in type 1 muscle fibers giving a checkerboard pattern with the lighter reticular end product in type 2 muscle fibers and a pale pink/purple background. Tubular aggregates are intensely stained.

Notes

a. Dithiothreitol attacks electrodes. Avoid contact totally when adjusting pH of solution.
b. For the control use 4 mg inosine 5′- monophosphate instead of AMP.

Demonstration of myophosphorylase

Myophosphorylase is the muscle isoform of glycogen phosphorylase which catalyses the rate-limiting step in glycogenolysis by breaking down glycogen to release glucose 1-phosphate. Glycogen storage disease type V (McArdle's disease) is caused by deficiency of myophosphorylase.

A positive reaction gives colors through brown to deep blue/black depending on the chain length. A negative reaction gives a yellow color. The reaction works best if there is natural myofibrillary glycogen present to start the process. The corollary of this is that an absence of a phosphorylase reaction does not automatically mean that there is no phosphorylase present.

Myphosphorylase technique

Sections

Unfixed cryostat.

Solutions

0.1M acetate buffer pH 5.8–5.9

0.1M sodium acetate	85 ml
0.1M acetic acid	15 ml

Check that pH is 5.8–5.9. Adjust pH as necessary.

Insulin (20 units/ml)

Phosphorylase incubating medium

Distilled water	50 ml
0.1M acetate buffer pH 5.8–5.9	50 ml
74OP spirit	25 ml
Glucose 1-phosphate (potassium salt)	250 mg
Adenosine 5-monophosphate	50 mg
Glycogen	10 mg
Insulin (20 units/ml)	5 drops

Thoroughly mix all the ingredients together and adjust pH to 5.8–5.9.

Gram's iodine

Distilled water	300 ml
Potassium iodide	3 g
Iodine	1 g

Dissolve the potassium iodide in the distilled water. Add the iodine and mix well. Then mix 1 ml of this with 9 ml distilled water to produce a 10% solution.

Iodine/glycerol mountant (1:4 dilution)

Glycerol	15 ml
Gram's iodine	5 ml

Mix both together well. The mountant keeps well at room temperature.

Method

The technique is temperature sensitive and requires careful temperature checking prior to and during staining. Set incubator to 37°C ±1 °C.

1. Filter on pre-warmed (37°C) phosphorylase incubating medium to frozen sections and incubate in a wet chamber at 37°C for 2 hrs.
2. Rinse in distilled water.
3. Develop color in 10% Gram's iodine for 1-5 min.
4. Mount wet from iodine in iodine/glycerol mountant.
5. Seal the edges of the cover-slip in ringing media or clear nail polish.

Results

Sites of phosphorylase activity, shades of blue/black.

Notes

a. Use a normal muscle section as a control and repeat the technique.

b. The section will fade in 2-3 days but may be reactivated by removing the cover-slip and re-developing of the color in Gram's iodine, following steps 3-5 of the method, if required.
c. Alternatively, to avoid fading, at stage 4 (following developing in Gram's iodine), rinse slides quickly with distilled water and allow to air dry, clear and mount in either Micromount or DPX.

Demonstration of phosphofructokinase

Phosphofructokinase (PFK) is another glycolytic enzyme which catalyzes the transfer of a phosphate group from ATP to fructose-6-phosphate (F6P), generating fructose-1,6-bisphosphate. It is a key regulator of glycolysis.

PFK deficiency, Tarui's disease, is a glycogen storage disorder characterized by weakness with spasms and cramping on exercise.

Phosphofructokinase technique

Sections

Unfixed cryostat.

Solutions

PFK incubating medium

20 mmol sodium arsenate	8.0 ml
10mmol d-fructose-6-phosphate*	3.2 ml
10mmol nicotinamide adenine dinucleotide*	1.6 ml
10mmol adenosine triphosphate*	1.6 ml
40mmol magnesium sulphate	0.4 ml
Nitro blue tetrazolium (NBT)	6.4 mg
Distilled water	1.2 ml

*These components must be made fresh prior to making up the solution fresh.

Adjust pH to 7.0.

Method

1. Filter on pre-warmed (37°C) PFK medium and incubate in a wet chamber at 37°C for 1–2 hours.
2. Rinse in distilled water.
3. Take through ascending 30%, 60% and 90% acetone to remove excess red mono formazan produced in the reaction for a few seconds each.
4. Dehydrate, clear and mount in Pertex.

Results

Sites of PFK activity, shades of blue / purple.

Acknowledgments

Thanks to Sebastian Brandner for providing us with his protocol for methylene blue-azure II-basic fuchsin staining and to Patrick Elliott of the Medical Illustration Department, Royal Hallamshire Hospital, Sheffield, for his illustration depicted in Fig. 18.1. We are grateful to Dr Gabriele De Luca for advice regarding applying the Palmgren method to cryostat sections, and to the neuropathology laboratories of the John Radcliffe Infirmary (Oxford), Hull Royal Infirmary and Royal Hallamshire Hospital (Sheffield) for various muscle techniques.

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Immunohistochemical and immunofluorescent techniques

19

Tracy Sanderson • Graeme Wild • Ann Michelle Cull • Jennifer Marston • Greg Zardin

Introduction

The introduction of prognostic and predictive markers in immunohistochemistry (IHC) has made a tremendous beneficial impact on patient diagnosis and management. In the 1940s the demonstration of tissue constituents using an antibody labeled with a fluorescent dye was first described by Coons et al. (1941). This original work used a method called direct immunofluorescence, where the specific primary antibody was conjugated directly with the fluorochrome and viewed under a fluorescent microscope. Indirect immunofluorescence was introduced by Weller and Coons (1954). It is distinct from the direct method as the specific primary antibody is unlabeled and is detected using a fluorochrome-labeled anti-species specific immunoglobulin antiserum.

Following on from these original discoveries there has been a gradual development of IHC methodologies, which have allowed the identification of specific and highly selective cellular epitopes in formalin fixed, paraffin wax processed tissues with an antibody and appropriate labeling system.

Many of the immunofluorescence techniques were further enhanced with the introduction of enzymes as labels. Cells which have been labeled with an enzyme such as horseradish peroxidase conjugated to an antibody are visualized with an appropriate chromogen such as diaminobenzidine (DAB) (Nakane & Pierce, 1966). The nuclear detail is visualized by counterstaining with a traditional stain such as hematoxylin. This permits the simultaneous evaluation of both specific IHC and morphological detail. Further developments saw the introduction of the peroxidase-anti-peroxidase (PAP) technique by Sternberger et al. (1970), followed by alkaline phosphatase labeling by Engvall and Perlman (1971) and then Cordell et al. (1984) described the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique. In 1977, Heggeness and Ash proposed the use of avidin-biotin for immunofluorescence; this technique was then modified by Guesden et al. (1979) and Hsu et al. (1981) who used a horseradish peroxidase label. Avidin-biotin labeling was superseded by streptavidin-biotin labeling, and was one of the more popular techniques used. Labeled polymer detection systems are now the standard choice for most diagnostic laboratories.

Many antibodies are now available to identify epitopes which survive formalin fixation and processing to paraffin wax. In the 1970s it was thought that routine paraffin wax processing destroyed many epitopes, and that certain antigens could never be demonstrated in paraffin wax sections. It was later found that many antigens are not lost, but masked by the processes involved in formalin fixation and paraffin wax processing.

The introduction of heat-induced epitope retrieval techniques allowed the demonstration of many epitopes in formalin fixed, paraffin wax processed tissue, which previously could only be performed on frozen sections. For example, the proliferation antigen Ki67 and T-cell antigens CD2, CD4, CD5 (Fig. 19.1), CD7 and CD8 can all now be reliably demonstrated in paraffin wax sections. Furthermore, other antibodies such as those directed against the leukocyte common antigen (clones PD7/2B11) and the CD20 antigen (clone L26) produce enhanced staining after

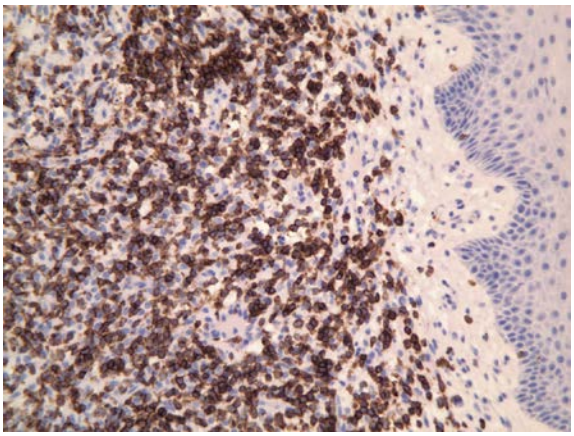

Fig. 19.1 CD5 demonstration in a formalin-fixed paraffin-embedded section from a nasal biopsy showing extra-nodal T-cell lymphoma.

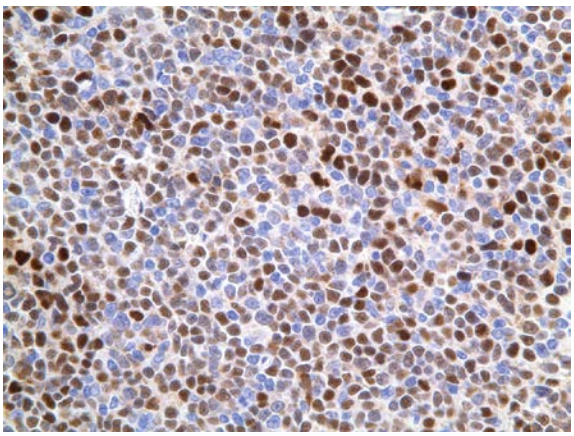

Fig. 19.2 Formalin-fixed paraffin-embedded section of lymph node, showing cyclin D1 expression in a mantle cell lymphoma. Antigen retrieval with the pressure cooker using citrate buffer, pH 6.0 and Thermo rabbit monoclonal (clone SP4) primary antibody.

citrate buffer (pH 6.0) heating. Surprisingly, heat pretreatment allows for considerably greater dilution factors. The demonstration of antigens such as cyclin D1 (with clone DCS-6), on the other hand, is better in a high pH solution (Tris-EDTA, pH 10.0). The use of the cyclin D1 rabbit monoclonal antibody (clone SP4) however, produces good results using citrate buffer at pH 6.0 (Fig. 19.2). In short, antigen retrieval and demonstration is possible with modulation of section treatments.

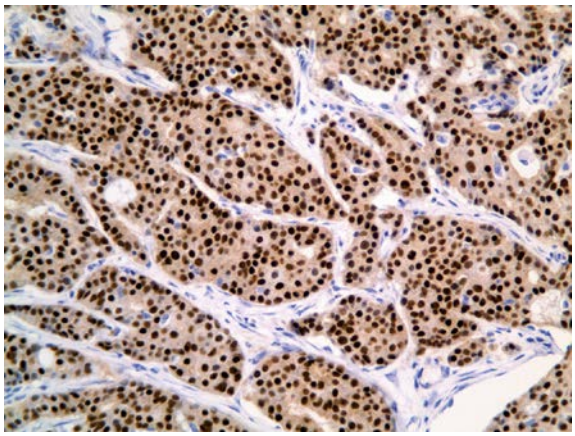

Fig. 19.3 Formalin-fixed paraffin-embedded section of breast carcinoma showing strong expression of estrogen receptor. Pressure cooker antigen retrieval for 2 minutes using Vector antigen unmasking fluid.

As a result of these developments the use of frozen sections for IHC analysis has been greatly reduced, but is still useful in some diagnostic areas, such as determining the nature of protein deposits in skin and renal diseases, as well as identifying bacteria in infected material.

All of these technical advances have led to IHC becoming an invaluable aid in the diagnosis of cancer. Many tests are now referred to, or recommended in, national reporting guidelines such as those set out by the Royal College of Pathologists (see further reading). The increasing use of prognostic and predictive markers permits the pathologist to make diagnostic evaluations which directly influence patient management (Fig. 19.3).

The demand for this aspect of histological analysis has led to dramatic increases in workload globally; most laboratories now use some form of automated immunostainer to process the large volumes of slides with which they are presented.

One of the increasing challenges to face the IHC laboratory is the move towards personalized medicine. Over the last few years there has been an increase in the requirement for molecular tests to be performed on samples either alongside or after IHC has been completed. With the ongoing developments in understanding the biology of different tumor types, many genetic factors have been

identified which are known to have an effect on the response to certain treatments. These have an impact on which therapies the patient is offered. Pharmaco-diagnostics or companion testing is likely to increase over the next few years, where an IHC test is developed alongside the drug to be used for treatment. Not only will this present new technical challenges to the immunohistochemist as discussed by Taylor (2014), it also means there could be competing demands for material from these diagnostic samples. Many of these are now core biopsy specimens and conservation of this limited amount of tissue will become even more important.

Herceptin®/trastuzumab was one of the first examples of a companion test, where the patient has to have a positive result to be eligible for the drug treatment. More recently PDL-1 testing has been linked to drugs available for the treatment of lung cancers (Shien et al., 2016; Shimoji et al., 2016).

As the reliance on IHC has developed, the need for effective monitoring of test performance has grown. Not only should there be robust internal quality control procedures, many diagnostic laboratories now participate in external quality assurance schemes as well.

Immunohistochemistry theory

Definitions

Immunohistochemistry (IHC)

A technique for identifying cellular or tissue constituents, antigens, by means of antigen-antibody interactions. The site of antibody binding is identified either by direct labeling of the antibody or by use of a secondary labeling method.

Antigen

An antigen is a molecule which induces the formation of an antibody and bears one or more antibody-binding sites. These are highly specific topographical regions composed of a small number of amino acids or monosaccharide units known as antigenic determinant groups or epitopes.

Antibody

Antibodies belong to the class of serum proteins known as immunoglobulins. The terms antibody and immunoglobulin are often used interchangeably. They are found in blood and tissue fluids, as well as many secretions. The basic unit of each antibody is a monomer; antibodies can be monomeric, dimeric, trimeric, tetrameric, or pentameric in format. The monomer is composed of two heavy and two light chains. If it is cleaved with enzymes such as papain and pepsin, two fragment binding antigens (Fab) fragments and a crystallizable fragment (Fc) are produced. They are formed in the humoral immune system by plasma cells, the end cell of B-lymphocyte transformation, after recognition of a foreign antigen. There are five types of antibody found in the blood of higher vertebrates: IgA, IgD, IgE, IgG, and IgM.

IgG is the most common and frequently used antibody for IHC. The IgG molecule is composed of two pairs of light and heavy polypeptide chains linked by disulfide bonds to form a Y-shaped structure. The terminal regions of each arm vary in amino acid sequence and are known as 'variable domains'. This variability in amino acid content provides specificity for a particular epitope and enables the antibody to bind specifically to the antigen against which it was raised.

Antibody-antigen binding

The amino acid side-chains of the variable domain of an antibody form a cavity-like site which is geometrically and chemically complementary to an antigen epitope molecule, as described by Capra and Edmundson in 1977. The analogy of a lock (antibody) and key (antigen) has been used, and the precise fit required explains the high degree of antibody-antigen specificity seen. The associated antibody and antigen are held together by a combination of hydrogen bonds, electrostatic interactions, and van der Waals' forces.

Affinity

Affinity is the three-dimensional fit of the antibody to its specific antigen, and is a measure of the binding strength between the antigenic epitope and its specific antibody-combining site.

Avidity

Avidity is a related property referring to the heterogeneity of the antiserum which will contain various antibodies reacting with different epitopes of the antigen molecule. A specific but multivalent antibody is less likely to be removed by the washing process than a monovalent antibody. Avidity therefore is the functional combining strength of an antibody with its antigen.

Antibody specificity

This is the characteristics of an antibody to bind selectively to a single epitope on an antigen.

Sensitivity

This is the relative amount of antigen which an IHC technique is able to detect. A technique with high sensitivity is able to detect smaller amounts of antigen than a technique with low sensitivity. If used to detect the same amount of antigen, the technique with high sensitivity would produce a larger signal than a method with low sensitivity.

Production of primary reagents

Polyclonal antibodies

Polyclonal antibodies are produced by immunizing an animal with a purified specific molecule, an immunogen, bearing the antigen of interest. The animal will mount a humoral response to the immunogen and the antibodies produced can be harvested by bleeding the animal to obtain immunoglobulin-rich serum. It is understood that numerous clones of plasma cells will be activated to produce the polyclonal antibodies.

Each clone will produce an antibody with a slightly different specificity to the variety of epitopes present on the immunogen. A polyclonal antiserum is therefore a mixture of antibodies to different epitopes on the immunogen. Some of these antibodies may cross-react with other molecules and will need to be removed by absorption with the appropriate antigen. The antiserum will probably contain antibodies to impurities in the immunogen. Antibodies raised against the contaminating immunogens are often of low titer and/or affinity, and can be diluted out to zero activity for immunolabeling. There is a high possibility that a wide spectrum of naturally occurring antibodies will be present in the host animal as a response to previous antigen challenges. Serum removed from the animal, before injection of the immunogen, is therefore important as a negative or pre-immune control. For precise details of polyclonal antibody production see De Mey and Moeremans (1986).

Monoclonal antibodies

The development of the hybridoma technique by Kohler and Milstein (1975) to produce monoclonal antibodies has revolutionized IHC by increasing enormously the range, quality and quantity of specific antisera. Detailed descriptions of the technique have been given by Gatter et al. (1984) and Ritter (1986).

The method combines the ability of a plasma cell (transformed B lymphocyte) to produce a specific antibody with the in vitro immortality of a neoplastic myeloma cell line; a hybrid with both properties can be produced. With the technique of cloning, this cell can be grown and multiplied in cell culture theoretically to unlimited numbers. By careful screening, hybrids producing the antibodies of interest, without cross-reactivity to other molecules, can be chosen for cloning. The original antigen does not need to be pure as hybrids reacting to unwanted antigens or epitopes can be eliminated during screening. The result is a constant, reliable supply of one pure monoclonal antibody with known specificity.

This approach to the production of monoclonals has dramatically increased the number of antibodies available for IHC and has allowed for further evolution with the ability to identify more antigens in paraffin wax sections. Detailed comparisons of the values and limitations of polyclonal and monoclonal antibodies have been given by Warnke et al. (1983) and Gatter et al. (1984).

For immunofluorescent techniques a preparation of highly purified or recombinant antigen is an absolute requirement for the production of monospecific antiserum having a high affinity and avidity. Antibody specificity can be determined by reacting it against the purified antigen used to immunize

the animal. It can also be tested against the unpurified source such as whole human serum, e.g. if the antibody is against a specific human protein. This checking for monospecificity can be achieved by gel diffusion, immunoelectrophoresis or passive hemagglutination, and should result in the production of one precipitin line in both the purified antigen and the unpurified source. Antibody concentrations of relatively high titer are required for conjugation with fluorochromes.

Labels

Enzyme labels

Enzymes are the most widely used labels in immunohistochemistry, and incubation with a chromogen using a standard histochemical method produces a stable, colored reaction end product suitable for the light microscope. In addition, the variety of enzymes and chromogens available allow the user a choice of color for the reaction end product.

Horseradish peroxidase (HRP) is the most widely used enzyme, and in combination with the most favored chromogen, 3,3α-diaminobenzidine tetrahydrochloride (DAB), it yields a crisp, insoluble, stable, dark brown reaction end product (Graham & Karnovsky, 1966). DAB is classed as a hazardous chemical and has been reported to be a potential carcinogen (Weisburger et al., 1978).

Horseradish peroxidase is commonly used as an antibody label for several reasons:

- Its small size does not hinder the binding of antibodies to adjacent sites.
- The enzyme is easily obtainable in a highly purified form and therefore the chance of contamination is minimized.
- It is a stable enzyme and remains unchanged during manufacture, storage and application.
- Any endogenous activity is easily quenched.

Other chromogens are available, including: 3-amino-9-ethylcarbazole (Graham et al., 1965; Kaplow, 1975), which gives a red final reaction product; 4-chloro-1-naphthol (Nakane, 1968), a blue final reaction product; Hanker-Yates reagent (Hanker et al., 1977), a dark blue product and α-naphthol pyronin (Taylor & Burns, 1974), a red-purple final reaction product. Many of these contain hazardous reagents and have now largely been superseded by commercial chromogens available in kit form.

Vector Laboratories, for example produce a wide range of different colored chromogens suitable as alternatives to DAB which can be used for multilabeling techniques. These include Vector Red, Vector Blue, Vector VIP (purple) and BCIP/NBT(blue/violet).

It should be noted that some of these chromogens produce reaction products which are soluble in alcohol and xylene, and therefore the sections require aqueous mounting. Commercial products are now available which have improved preservation qualities and resolution compared with the traditional aqueous mountants. After drying in a hot oven, these mountants give a hard permanent covering of the section. Other commercially available permanent mounting media which are non-aqueous and both toluene and xylene free are also available (Vector Laboratories VectaMount™), and they provide a permanent preparation for use with enzyme substrates such as Vector Red, Vector VIP and BCIP/NBT.

Endogenous peroxidase activity is present in a number of sites, particularly neutrophil polymorphs and other myeloid cells. Blocking procedures may be required, the hydrogen peroxide-methanol method (Streefkerk, 1972) being the most popular. Care should be taken with certain antigens, notably CD4, where too long an incubation in the blocking solution or too high a concentration of hydrogen peroxide can significantly diminish staining on formalin fixed, paraffin wax embedded tissue. Performing the peroxidase block after the binding of the primary antibody to the tissue antigen is to be recommended for antibodies such as CD4.

Calf intestinal alkaline phosphatase is the most widely used alternative enzyme tracer to horseradish peroxidase, particularly since the development of the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method by Cordell et al. (1984) Fast red TR used with naphthol AS-MX phosphate sodium salt gives a bright red reaction end product

which is soluble in alcohol. New fuchsin has been reported as giving a permanent insoluble red product (Malik & Daymon, 1982) when mounted in resinous mountant, but it is the experience of many workers in the field that the resistance of the reaction product to resinous mounting is inconsistent.

Endogenous alkaline phosphatase activity is usually blocked by the addition of levamisole to the substrate solution. Levamisole selectively inhibits certain types of alkaline phosphatase, but not intestinal or placental, when used at a concentration of 1 mM. Twenty percent glacial acetic acid is a better blocker of endogenous alkaline phosphatase activity as it inhibits all types of alkaline phosphatase.

Other labels such as glucose oxidase, bacterial-derived β-D-galactosidase, colloidal gold and silver metals have been used in the past, but failed to find a routine use in the diagnostic laboratory. Colloidal gold has a much wider use in the electron microscope field.

Fluorescent labels

Fluorochromes are fluorescent labels which when conjugated to the antibody absorb ultraviolet or visible light of a particular wavelength to reach an unstable excited state as its electrons gain energy. The fluorochrome subsequently emits light of a different, usually longer, wavelength to that of the excitation light as the electrons return to their ground state.

Serum proteins have differing capacities to combine or conjugate with fluorochromes. Immunoglobulins, in particular, have less affinity for fluorescein isothiocyanate (FITC) than other more 'negatively charged' proteins such as albumin and β-proteins. These latter molecules when conjugated can also combine with tissue components via electrostatic forces and give high levels of non-specific staining. Purified immunoglobulin free from other serum proteins is therefore a pre-requisite for conjugation. Immunoglobulin molecules are composed of heavy and light chains. The heavy chains identify the isotype of the antibody and the light chains are common to all immunoglobulin types. Consequently, an antibody raised to a particular immunoglobulin type may also cross-react with all other immunoglobulin types due to the presence of antibodies to the light chains. These contaminating light chain antibodies should be removed by absorption against free light chains and leave only antibody reacting against the heavy chain component, i.e. γ, α or μ chain specific.

Conjugates may be prepared from:

1. Immunoglobulin rich fractions of serum prepared by salting-out procedures.
2. Chromatographically prepared fractions, usually on diethyl aminoethane (DEAE) ion exchange columns, consisting mainly of IgG.
3. Pure IgG fractions obtained by immunoabsorption on affinity chromatography columns.
4. F(Ab)2 fractions of IgG obtained by proteolytic cleavage of purified IgG which has been obtained as in 3 above.

The four methods are progressive in terms of the purity of their antibody preparation, and each can be used with success in different situations. For most routine applications, reagents prepared using purification methods 1 or 2 are adequate, particularly when they can be used at high dilution. Reagents prepared with method 2 are useful when background staining is a problem. Conjugates made from F(Ab)2 fragments are used when the binding of the conjugated antibody to Fc receptors is to be avoided. They may also be useful in double staining techniques where cross-reactions between antibodies produced in different species are a problem.

The absorption and emission characteristics of several commonly used fluorochromes are summarized in Table 19.1 (Allan, 2000). FITC is the most widely used fluorochrome in immunofluorescent microscopy. It has a wide absorption spectrum which covers the ultraviolet to blue light range and has a characteristic apple-green emission. An advantage of FITC is that the apple-green fluorescence is rarely seen as autofluorescence in mammalian tissues. Rhodamine absorbs maximally in green light and has an orange-red emission light; it can be used in a two color technique where two different antigens can be identified on the same section by antibodies conjugated with FITC or rhodamine.

The conjugation of a fluorochrome with an antibody can be a complex reaction and is dependent

Table 19.1 Spectral characteristics of commonly used fluorochromes

Fluorochrome	Absorption maximum (nm)	Emission maximum (nm)	Observed color
Fluorescein (FITC)	494	518	green
Rhodamine (TRITC)	550	580	red
Texas Red™	595	615	red
R-Phycoerythrin (PE)	565	575	orange/red

Protein localization by fluorescence microscopy. (Allan, V.J., 2000, Oxford University Press, Oxford).

on the type of fluorochrome. FITC and rhodamine can be linked covalently to free terminal amino and carboxyl groups, free amino groups on lysine side chains and free carboxyl groups in aspartic and glutamic acid residues. The reactions occur at pH 9.5 and the degree of conjugation is both time and temperature dependent. The ideal fluorochrome to antibody ratio is between 2 and 4:1.

Over-conjugation of the antibody will give high background staining as the molecules have a net negative charge and will bind to tissue non-specifically. This can also result in poor reactivity of the antibody due to interference with antigen binding sites. Under-conjugation gives a preparation which will produce unsatisfactory low-level fluorescence.

Free chromophore in the conjugate preparation must be removed to prevent non-specific staining. The free chromophore can be removed by dialysis against 0.15 M sodium chloride at 4°C. The presence or absence of fluorescence in the dialysate can be seen under UV examination. An alternative is to use gel filtration column chromatography using Sephadex G50. A method for testing commercial conjugate preparations for free dye is described by Johnson and Holborrow (1986).

It is good laboratory practice to evaluate the sensitivity and specificity of all antisera and conjugates used in immunofluorescence. In assessing the sensitivity of the reagents, a checkerboard test will indicate the optimal working dilution for a particular antibody or conjugate. For indirect immunofluorescence, serial dilutions of the antiserum or conjugate are tested against serial dilutions of the unlabeled primary antibody and the tissue sections assessed for the least amount of background fluorescence which still allows identification of the target antigen. The conjugate working dilution in direct immunofluorescence can be determined by examining serial dilutions of the conjugate on a tissue section containing known protein deposits e.g. renal sections from an IgA nephropathy or skin sections from known pemphigus or pemphigoid patients.

Specificity checking of a working strength conjugate on a tissue section with known deposits shows whether or not it will cross-react with other antigens. Anti-IgG γ-chain-specific conjugate for example, should not react with other classes of immunoglobulin such as IgA, IgM or kappa/lambda (κ/λ) light chains.

Some anti-animal immunoglobulin specific conjugates used in indirect immunofluorescence may cross-react with human immunoglobulins. In such cases it is essential that the cross-reacting antibodies are removed by absorption with human immunoglobulin. Cross-reactivity can be assessed by incubating the conjugated antibody directly on a tissue section containing human immunoglobulin and examining the slide for fluorescence.

Radiolabels

The use of radioisotopes as tracers requires autoradiographic facilities, and developed from the need for quantitation in IHC. Techniques involving the use of radioisotopes as tracers have been discussed by Hunt et al. (1986) but are not used in the routine diagnostic laboratory.

Immunohistochemical methods

There are numerous IHC staining techniques which may be used to localize and demonstrate tissue antigens. The selection of a suitable technique

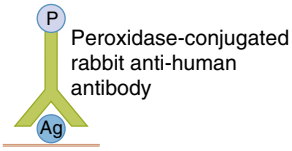

Fig. 19.4 Schematic representation of the direct IHC technique using HRP.

should be based on parameters such as the type of specimen under investigation, the type of preparation used e.g. frozen sections, paraffin wax sections, resin sections or cytological preparations, and the degree of sensitivity required.

Traditional direct technique

The primary antibody is conjugated directly to the label. The conjugate may be either a fluorochrome or an enzyme (Fig. 19.4). The labeled antibody reacts directly with the antigen in the histological or cytological preparation. The technique is quick and easy to use but it provides little signal amplification and lacks the sensitivity achieved by other techniques. It is mainly confined to the demonstration of immunoglobulin and complement in frozen sections of skin and renal biopsies. Low levels of antigen present in certain tumors may not be demonstrated by this technique and this could be crucial for an accurate and comprehensive diagnosis.

Two-step indirect technique

A labeled secondary antibody directed against the immunoglobulin of the animal species in which the primary antibody has been raised visualizes an unlabeled primary antibody (Fig. 19.5). Horseradish peroxidase labeling is most commonly used, together with an appropriate chromogen substrate. The method is more sensitive than the traditional direct technique because multiple secondary antibodies may react with different antigenic sites on the primary antibody, thereby increasing the signal amplification. The technique offers versatility as the same labeled secondary antibody can be used with a variety of primary antibodies raised from the same animal species.

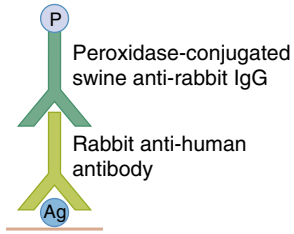

Fig. 19.5 Schematic representation of indirect IHC technique using HRP.

Polymer chain two-step indirect technique

This technology uses an unconjugated primary antibody, followed by a secondary antibody conjugated to an enzyme (horseradish peroxidase) labeled polymer (dextran) chain (Fig. 19.6). This dextran chain has up to 70 enzyme molecules and 10 antibody molecules attached. Conjugation of both anti-mouse and anti-rabbit secondary antibodies enables the same reagent to be used for both monoclonal (rabbit and mouse) and polyclonal (rabbit) primary antibodies. The method is biotin free and therefore does not react with endogenous biotin. In addition to being quick, reliable and easily reproducible, the technique offers great sensitivity. The technique is also useful for multi-color staining on single slide preparations. This technique is now the most commonly used method in routine diagnostic use. There is a wide choice of commercially available polymer kits, e.g. EnVision™+ and FLEX+ from Dako, Novolink and Bond Polymer Refine from Leica, Immpress™ from Vector Laboratories, Excel + from Menarini and ultraVIEW from Ventana.

Unlabeled antibody-enzyme complex techniques (PAP and APAAP) and Immunogold silver staining technique (IGSS)

These methods are rarely found now in the diagnostic setting but descriptions of these techniques can be found in previous editions of this publication.

(Strept)avidin-biotin techniques

With the introduction of automation and the indirect polymer-based techniques, the labeled streptavidin-biotin method has less use in diagnostic IHC. This is

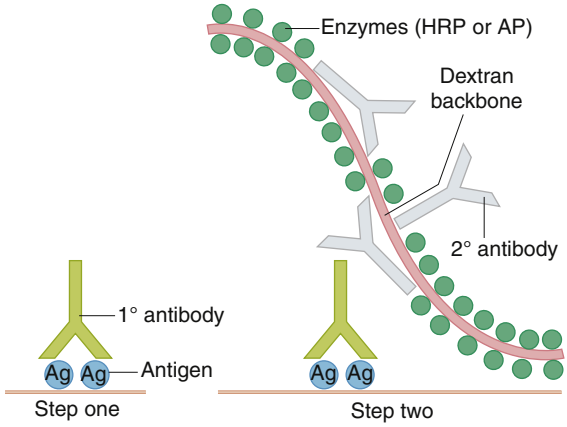

Fig. 19.6 Schematic representation of the polymer chain two-step indirect IHC technique using HRP (redrawn with permission from Dakocytomation™ Systems).

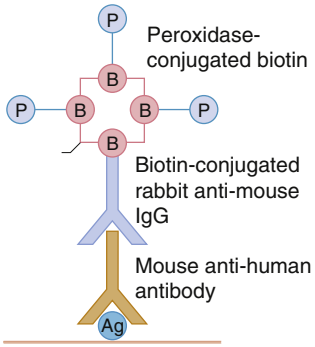

Fig. 19.7 Schematic representation of the avidin-biotin IHC technique using HRP.

a three-step technique, which has an unconjugated primary antibody as the first layer, followed by a biotinylated secondary antibody (raised against the species of the primary animal). The third layer is either a complex of enzyme-labeled biotin and streptavidin, or enzyme-labeled streptavidin (Fig. 19.7). The enzyme can be either horseradish peroxidase or alkaline phosphatase, used with a chromogen of choice.

These methods rely on the marked affinity of the basic glycoprotein avidin (MW 67 kDa) for the small water-soluble vitamin biotin (MW 244 Da). Avidin has two distinct disadvantages when used in IHC. Firstly it has a high isoelectric point of approximately 10 and is therefore positively charged at neutral pH. As a result, it may bind non-specifically to certain negatively charged structures such as cell nuclei. Secondly avidin, being a glycoprotein, also has a tendency to react with lectins via the carbohydrate moiety, again causing non-specific staining. Streptavidin (MW 60 KDa) has now largely replaced the use of avidin in IHC detection techniques as it overcomes these problems. Avidin and biotinylated reagents can also be used in indirect immunofluorescence.

Streptavidin can be isolated from the bacterium *Streptomyces avidini*, and like avidin it has four high-affinity binding sites for biotin. However, in practice due to the molecular arrangement of these binding sites, fewer than four biotin molecules actually bind. Biotin (vitamin H) is easily conjugated to antibodies and enzyme markers. Up to 150 biotin molecules can be attached to one antibody molecule, often with the aid of spacer arms. By spacing the biotins, the large streptavidin has room to bind and maximize its strong affinity for biotin. The streptavidin-biotin technique can employ either enzyme label bound directly to the streptavidin (Guesden et al., 1979); alternatively, the enzymes are biotinylated and the biotinylated label, forming the streptavidin-biotin complex (Hsu et al., 1981), occupies 75% of the streptavidin-binding sites. Usually, the latter is commercially supplied as two separate reagents, biotinylated label and streptavidin, and they are added together 30 minutes before use in order for the complex to form fully. Careful stoichiometric

control ensures that some binding sites remain free to bind with the biotinylated secondary antibody. As a large number of biotins can be attached to a single antibody, then numerous labeled streptavidin molecules may be bound on top. This produces increased sensitivity compared to the previously described enzyme techniques and allows a higher dilution of the primary antibody. Tissues rich in endogenous biotin such as liver and kidney will require the use of an avidin/biotin block before applying the primary antibody.

Amplification methods

Following the improvements in the quality of antibodies and polymer based visualization reagents, the use of amplification systems such as biotinylated tyramide signal amplification and biotin-free catalyzed signal amplification (CSA II) are not routinely required in the diagnostic laboratory. A description of the methodologies can be found in previous editions of this publication.

Unmasking of antigen sites

The concept that antigens can be masked by the chemical processes involved in formalin fixation and paraffin wax processing, and that some form of unmasking of these antigens is required, dates far back into the history of IHC (Brandtzaeg, 1983). When formalin based fixatives are used, intermolecular and intra-molecular cross-linkages are formed with certain structural proteins. These are responsible for the masking of the tissue antigens. This adverse effect has been thought to be the result of the formation of methylene bridges between reactive sites on tissue proteins (Bell et al., 1987; Mason & O'Leary, 1991). These reactive sites include primary amines, amide groups, thiols, alcoholic hydroxyl groups and cyclic aromatic rings. The degree of masking of the antigenic sites depends upon the length of time in fixative, temperature, concentration of fixative, and the availability of other nearby proteins able to undergo cross-linkage.

It must be remembered that tissue sections are unique to the laboratory of origin. Differences in the type and duration of fixation, together with variations in tissue processing schedules, reagents, and the manner by which sections are dried after microtomy, are important considerations. As a consequence, it is often found that one method of antigen unmasking may provide optimal results for one laboratory, but not for another. Therefore, each method of antigen unmasking should be carefully evaluated using the laboratory's own material. Indeed, digestion or heating times may need to be slightly modified to the times stated in antibody data sheets or published methodologies.

The majority of diagnostic laboratories now use automated IHC staining systems, some of which have the ability to perform on-board antigen retrieval, using either enzymes or more commonly heat. Most platforms offer the user the choice of two different antigen retrieval buffers at different pH values, and possibly with variable heating times. Depending on the system used, the de-waxing and antigen retrieval may be separate steps, or combined by using a single reagent.

Where laboratories still employ manual methods for antigen unmasking they may include:

- Proteolytic enzyme digestion.
- Microwave oven irradiation.
- Pressure cooker heating.
- Water bath heating.
- Steamer heating.
- Formic acid (used mainly by neuropathology laboratories).

Before antigen unmasking pretreatments are employed, the sections are de-waxed, rinsed in alcohol then rinsed in water.

Proteolytic enzyme digestion

Pretreating formalin-fixed routinely processed paraffin wax sections with proteolytic enzymes to unmask certain antigenic determinants was described by Huang et al. (1976), Curran and Gregory (1977) and Mepham et al. (1979). The most popular of these employed today are trypsin and protease, but others such as chymotrypsin, pronase, proteinase K and pepsin may also be used. The theory behind the unmasking properties of these proteolytic enzymes is not fully understood.

It is generally accepted however, that the digestion breaks down formalin cross-linking and hence the antigenic sites for a number of antibodies are exposed.

Proteolytic digestion can be detrimental to the demonstration of some antigens, occasionally producing false positive or false negative results. Digestion times need to be tailored to individual antibodies and to the fixation time. Under-digestion results in too little staining because the antigens are not fully exposed; over-digestion can produce false positive staining, high background levels and tissue damage. There can be a fine balance between under-digestion and over-digestion when using proteolytic enzymes. Duration of enzyme digestion, enzyme concentration, use of a coenzyme such as calcium chloride with trypsin, temperature and pH must be optimized to produce consistent high-quality IHC staining. Different batches of enzyme may vary in quality and each new batch of enzyme should be assessed prior to routine use. Enzymes produced specifically for IHC use are now widely available from commercial sources. These have been produced for use with automated immunostaining machines, are easy to use and give good consistent results.

The use of heat-induced epitope retrieval techniques has largely replaced proteolytic digestion. To demonstrate immunoglobulins and complement in formalin-fixed paraffin wax embedded renal biopsies and for a number of other individual antigens however, proteolytic digestion is still favored by many.

Heat-mediated antigen retrieval techniques

Heat-based antigen retrieval methods have brought a great improvement to the quality and reproducibility of IHC. There are multiple theories of the rationale behind antigen retrieval, firstly, cross-linkages between formalin and protein could be disrupted by heating above 100°C, or by strong alkaline treatment (Shi et al., 1991).

Another theory is that during formalin fixation inter-molecular, methylene bridges and weak Schiff bases form intra-molecular cross-linkages. These cross-linkages alter the protein conformation of the antigen, which may prevent it from being recognized by a specific antibody. It is postulated that heat-mediated antigen retrieval removes the weaker Schiff bases but does not affect the methylene bridges, so the resulting protein conformation is intermediate between fixed and unfixed.

Another possible theory was described by Morgan et al. (1997), who postulated that calcium coordination complexes formed during formalin fixation prevent antibodies from combining with epitopes on tissue-bound antigens. The underlying theory of calcium involvement is that hydroxymethyl groups and other unreacted oxygen-rich groups, e.g. carboxyl or phosphoryl groups can interact with calcium ions to produce large coordinate complexes which can mask epitopic sites by steric hindrance. The high temperature weakens or breaks some of the calcium coordinate bonds, but the effect is reversible on cooling because the calcium complex remains in its original position. The presence of a competing chelating agent at the particular temperature at which the coordinate bonds are disrupted, removes the calcium complexes. Evidence to support this theory comes from the chemical nature of some of the antigen retrieval reagents, e.g. citrate buffer and EDTA. In addition, it has been shown that the inclusion of calcium ions with an unmasking reagent inhibits its effectiveness (Morgan et al., 1994).

Microwave antigen retrieval

Shi et al. (1991) first established the use of microwave heating for antigen retrieval; the use of heavy metal salts posed a significant risk to the health and safety of the users. Gerdes et al. (1992) used microwave antigen retrieval with a non-toxic citrate buffer at pH 6.0 and demonstrated the Ki67 antigen which previously had been thought to be lost during formalin fixation and paraffin wax processing. The results were equivalent to those seen in frozen sections. Cattoretti et al. (1993) established microwave oven heating as an alternative to proteolytic enzyme digestion. The method improved the demonstration of well-established antibodies such as

Fig. 19.8 Domestic microwave oven.

CD45 and CD20 and enabled the demonstration of a wide range of new antibodies, such as CD8 and p53.

Numerous antigen retrieval solutions have been described, probably the most popular are 0.01 M sodium citrate buffer at pH 6.0 and 0.1 mM EDTA at pH 8.0. Although an expanding range of commercial antigen buffers at both high and low pH ranges is available, some are designed to improve the staining of specific antigens.

Most domestic microwave ovens are suitable for antigen retrieval and operate at 2.45 GHz, corresponding to a wavelength in vacuo of 12.2 cm (Fig. 19.8). Uneven heating and the production of hot spots have been reported by some workers using the microwave oven. However, by using a volume of buffer between 400 and 600 ml in a suitably sized microwave-resistant plastic container, the problems of uneven heating may be minimized. A batch of up to 25 slides in a plastic staining rack can be irradiated at one time and accurate, even antigen retrieval achieved. The actual heating time will depend on the following factors:

- Wattage of the oven. Most domestic ovens use a magnetron with an output between 750 and 1000 W. An important point to remember is that the output of the magnetron will decrease with age and frequency of use. The magnetron should be checked for efficiency annually.
- Choice of antigen retrieval buffer.
- Volume of buffer being used.
- Fixation of the tissues under investigation, i.e. fixative used and duration of fixation. This is an important factor, although not as critical as when using proteolytic enzyme digestion. Tissue fixed for extended periods of time will require extended irradiation times. Conversely, poorly fixed tissues may require a reduction in the heating time.
- Thickness of the tissue section: 3 µm sections require less antigen retrieval than 5 µm sections.
- Antigen to be demonstrated. Certain nuclear antigens may require increased heating times.

If extended heating times are used with a small volume of buffer, the buffer may need topping up with distilled or de-ionized water. This should be performed halfway through the total heating duration. At no stage should the sections be allowed to dry out during the antigen retrieval process.

Pressure cooker antigen retrieval

Norton et al. (1994) suggested the use of a pressure cooker as an alternative to the microwave oven. By using a pressure cooker, Norton et al. (1994) showed that the batch variation and production of hot and cold spots in the microwave oven could be overcome. Pressure cooking is said to be more uniform than other heating methods. A pressure cooker at 15 psi (10.3 kPa) reaches a temperature of around 120°C at full pressure. It is this increased temperature which appears to be a major advantage when unmasking certain nuclear tissue antigens, e.g. bcl-6, p53, p21, estrogen receptor and progesterone receptor. The demonstration of these antigens can sometimes be weak when using microwave antigen retrieval.

It is preferable to use a stainless steel domestic pressure cooker, because aluminum pressure cookers are susceptible to corrosion from some of the antigen retrieval buffers (Fig. 19.9). The pressure cooker should have a capacity of 4–5 liters, allowing a large batch of slides to be treated at the same time. As with the microwave oven, the use of charged microscope slides or strong adhesives such as Vectabond or APES is required to prevent section loss.

Fig. 19.9 Stainless steel pressure cooker and halogen hot plate.

Steamer

Although quite a popular method in some parts of the world, steam heating appears to be less efficient than either microwave oven heating or pressure cooking (Pasha et al., 1995). Times in excess of 40 minutes are sometimes required, but the method does have the advantage of being less damaging to tissues than the other heating methods. Commercially available rice steamers are adequate for this purpose.

Water bath

Kawai et al. (1994) demonstrated that a water bath set at 90°C was adequate for antigen retrieval. However, by increasing the temperature to 95–98°C, antigen retrieval was improved and the incubation times could be decreased. This technique has the advantage of being less damaging on the tissue sections because the temperature is set below boiling point. By using a lower temperature than other heating methods, the antigen retrieval buffer does not evaporate and expensive commercial antigen retrieval solutions can safely be reused. The method has the disadvantage that the antigen retrieval times are increased compared to other methods.

Advantages of heat pretreatment

Some antigens previously thought lost in routinely processed, paraffin wax embedded sections are now recovered by heat pretreatment. Many antigens are retrieved by uniform heating times, regardless of the length of fixation, e.g. up to several weeks in formal saline (Singh et al., 1993). The demonstration of heavy-chain immunoglobulins is more reliable and reproducible than when proteolytic digestion is employed. The dilution factors of some primary antibodies ascertained with traditional methods can be increased when using heat pretreatment.

Pitfalls of heat pretreatment

Care should be taken to prevent the sections drying after heating, as this destroys antigenicity. The boiling of poorly fixed material also damages nuclear detail. Fibrous and fatty tissues tend to detach from the slide; this can sometimes be overcome by increasing the drying temperature to 56°C and using Superfrost Plus microscope slides. Alternatively, Vectabond or APES-coated slides can be dipped in 10% formal saline for 1–2 minutes and air dried before picking up the sections. This tends to improve the adhesion, probably by adding more aldehyde groups to the slide surface. Not all antigens are retrieved by heat pre-treatment, and the range of staining of some primary antibodies, e.g. PGP9.5, a neuroendocrine marker, is altered (Langlois et al., 1994).

Commercial antigen retrieval solutions

There are numerous commercial antigen retrieval solutions available. They can either be specialized high pH solutions, recommended for certain antibodies, or lower, pH 6.0 for more general use. These solutions may be a mixture of different chemicals, such as citrate and EDTA. They offer advantages over the 'in house' retrieval solutions as they are ready to use, require no pH calibration and are fully certified to comply with laboratory accreditation procedures. However, they can be expensive.

Detection of low levels of antigen

Enhancement and amplification

The optimum dilution of primary antibody for diagnostic IHC is defined as the concentration of the primary antibody which gives the optimal specific

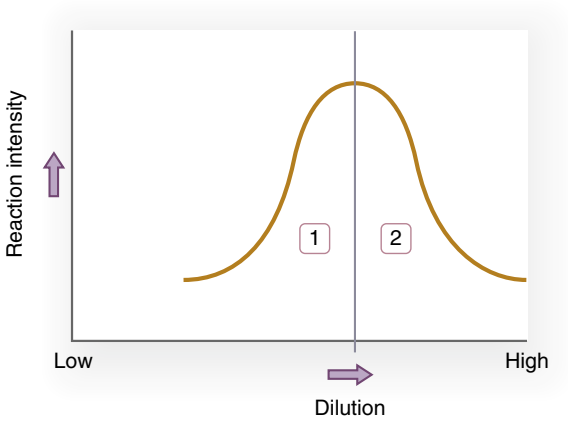

Fig. 19.10 Antibody dilution curve.

staining with the least amount of background staining. The optimal dilution will depend upon the type and duration of fixation. Serial dilutions of antibody will often give the distribution of reactivity shown in Fig. 19.10:

- Poor reaction in area 1 is due to steric hindrance of the labeling antibody accessing the primary antibody, the prozone effect. This is due to the primary antibody being too concentrated.
- Suboptimal reaction in area 2 is caused by inadequate presence of primary antibody i.e. the primary antibody is too diluted.

The optimum concentration of primary antibody is that measured below the apex of the peak, and the use of several control sections with varying expression of antigen will aid the determination of a correct working dilution of primary antibody for each particular laboratory. Inter-laboratory variations in the choice of fixative, duration of fixation, paraffin wax processing, section treatment and the IHC detection system used, make the dilution of primary antibody unique to the laboratory which produced the paraffin wax section and accounts for the great variation in the dilution of primary antibodies used from laboratory to laboratory.

The dilutions of primary antibodies and labeling systems for diagnostic IHC should be ascertained on material where the antigen levels are adequate but not excessive. Occasionally situations arise where some tumors shed much of their antigen, e.g. prostatic tumors often express less prostate specific antigen (PSA) than normal prostatic glands. Enhancement and amplification by modification of demonstration techniques may be required to identify low levels of antigens. This can be achieved by the following methods:

1. Increasing the concentration of the primary antibody. This can usually be accomplished with most monoclonals without significantly increasing the background staining as this type of antibody, especially in the form of tissue culture supernatant, does not contain any non-specific contaminants. Polyclonal antibodies can give excessive background problems and it is advisable to use a casein blocking solution, as described in the methods later in this chapter. Occasionally the addition of a small amount of detergent, e.g. 0.01% Tween, to the washes helps to reduce background staining. Further details on dealing with background staining appear later in the text.
2. Prolonging incubation with the primary antibody overnight, at 4–8°C or at ambient temperature, can enhance staining. Many immunohistochemists employ this methodology for their routine work because higher dilution of primary reagents is achieved, allowing costs to be reduced. Dilutions must not be excessive, otherwise low levels of antigen will not be detected, resulting in false negative staining.
3. Increasing the concentration of the bridge reagent beyond the optimal dilution, or repeated application of the bridge reagent, marginally increases the sensitivity of the avidin-biotin systems. Furthermore, in the case of the CD15 primaries, which are IgM subclass antibodies, LeBrun et al. (1992) reported that an IgM link, as opposed to a broad-spectrum immunoglobulin bridge reagent, improves the rate of detecting CD15-positive Reed-Sternberg and Hodgkin cells (Fig. 19.11). Charalambous et al. (1983) confirmed this work, but also indicated that when microwave antigen recovery was used in place of trypsin, further amplification was achieved.

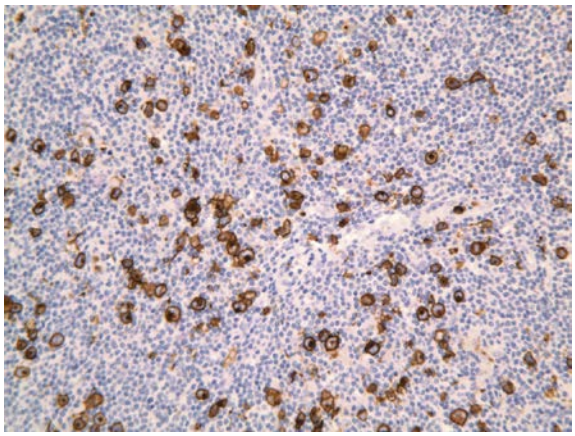

Fig. 19.11 CD15 demonstration in a formalin-fixed paraffin-embedded section of Hodgkin's disease.

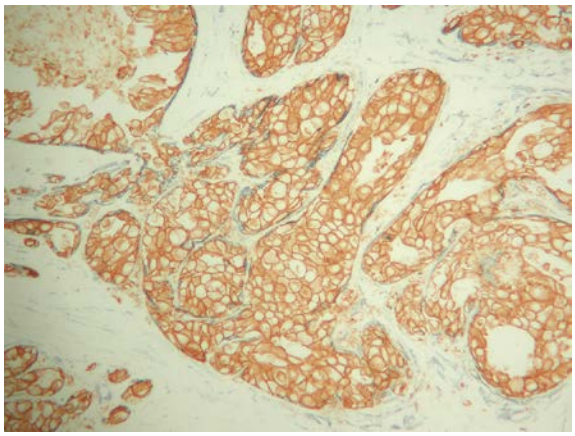

Fig. 19.12 Double IHC staining of formalin-fixed paraffin-embedded section of breast carcinoma shows HER2 membrane staining with DAB as the chromogen and smooth muscle actin stained with Vector SG chromogen.

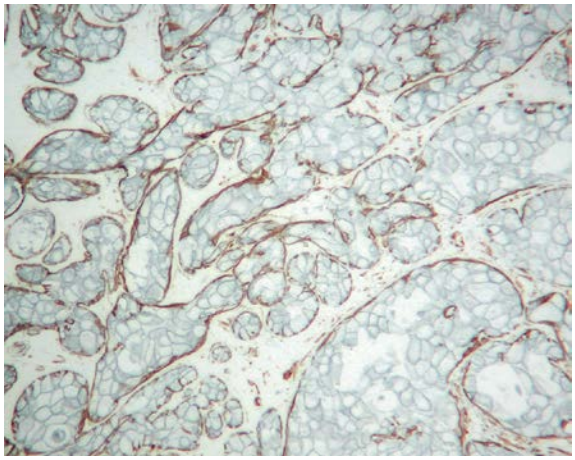

Fig. 19.13 Double IHC staining shows the smooth muscle actin demonstrated with DAB and the HER2 with Vector SG in a formalin-fixed paraffin wax-embedded section of breast carcinoma.

4. Chemical enhancement of the reaction end product of the peroxidase-DAB reaction may be employed and can be achieved by the addition of imidazole (Straus, 1982) or heavy metals such as copper or cobalt (Hsu & Soban, 1982).
5. Repeated applications of the bridge and label increase the sensitivity of the APAAP technique. Whilst the initial primary, bridge and label are incubated for 30 minutes each, the repeated applications of bridge and label require only 10 minutes each. After two such repeats enhancement is usually sufficient for most antibodies.
6. Changing the chromogen substrate, especially for alkaline phosphatase, gives a more intense reaction product. For example, nitro-blue tetrazolium is more intense than Fast Red and can be left on overnight to give probably the most intense reaction of all chromogens available today. The only drawback is that the blue-black reaction product does not contrast well with hematoxylin counterstaining. Improved commercial formulae of traditional substrates are superior to 'in house' formulae.

Multiple labeling techniques

The ability to label two or more different antigens in the same tissue section is playing a greater role in routine IHC (Figs. 19.12 and 19.13). The use of automated IHC staining systems, a greater range of substrate chromogens and the use of commercially available double labeling kits has provided a much more robust method than was previously available. The user has the choice of using the same detection system with different substrates, or of using different detection systems for individual substrates.

The methodology involves sequential staining of each of the primary antibodies; practice will help the user determine the optimum order of labeling and the optimum substrates/chromogens to use.

It is generally recognized that the primary antibody which gives the strongest final reaction product should be performed first (often with a DAB chromogen) followed by the weaker reaction. The choice of different enzyme-substrate combinations is important, as certain combinations work better and offer more contrast than others. The range of commercially available substrates from Vector Laboratories provides a wide choice. For example, when using alkaline phosphatase methods, the use of Vector Blue as the first substrate layer contrasts well when using Vector Red as the second substrate. For peroxidase methods the use of DAB as the first substrate contrasts well with Vector VIP as the second substrate.

Automation lends itself well to multiple labeling and this is facilitated by the availability of double labeling kits, e.g. Dako Envision™ DuoFLEX Doublestain system (HRP/AP) or the use of Leica Bond polymer Refine (DAB) with Bond Polymer Refine Red.

Immunohistochemistry in practice

Choice of technique

The choice of technique to suit the needs of each particular type of method is governed by the following important factors.

Fixation and paraffin wax block immunohistochemistry

A prerequisite for all routine histological and cytological investigations is to ensure preservation of tissue architecture and cell morphology by adequate and appropriate fixation (see Chapter 4). The most popular choice of fixatives for routine histology are formalin based, usually a 10% solution with the addition of phosphate buffers. The choice of fixative amongst pathologists was initially based on subsequent morphological appearance, and the clarity of established staining techniques using dyes which were the mainstay of diagnostic histopathology long before the advent of IHC. Most pathology teaching and learning is based on these traditional techniques, with all the artifacts they produce, and IHC has had to tailor itself to this type of material in order to become an effective diagnostic aid.

Prompt fixation of thin (3 mm) slices of tissue is essential to achieve consistent demonstration of tissue antigens. Delayed fixation or poor fixation may cause loss of antigenicity or diffusion of antigens into the surrounding tissue. Following fixation most material is routinely processed to paraffin wax to facilitate section cutting. It is therefore important to establish a fixation and processing procedure which retains good morphology and maximizes the ability of the immunohistochemist to identify the antigens which aid diagnosis. Ideally, if required, it should be possible, by prior arrangement with the surgeons and theater staff, to receive fresh specimens soon after surgery, in order that material can be selected for routine processing and frozen storage. The latter should be snap frozen using liquid nitrogen and stored at −80°C. This material can be used for preparing imprints for fluorescence in situ hybridization (FISH) techniques, as a source of RNA and DNA for molecular biology techniques, or for cutting frozen sections for the demonstration of antigens not readily demonstrated in paraffin wax sections. Recent advances have made it possible to perform some molecular techniques such as ISH on paraffin wax sections as well.

Retrospective studies are often hampered by a lack of knowledge of the duration of fixation. Banks (1979) indicated that prolonged fixation reduces immunoreactivity and, with formalin fixation this tends to occur over a period of weeks rather than days. Certain antibodies such as CD20 and CD45 are less affected by fixation times. According to Singh et al. (1993), microwave pretreatment enables the retrieval of antigens after formalin fixation even up to two years later. In general, the use of heat-mediated antigen retrieval has enabled a greater consistency of IHC staining over a wide range of different fixatives and fixation times (Figs. 19.14 and 19.15). Most individual laboratories will employ a single fixative used over a range of fixation times between 18 and 72 hours, a single standardized heat methodology can

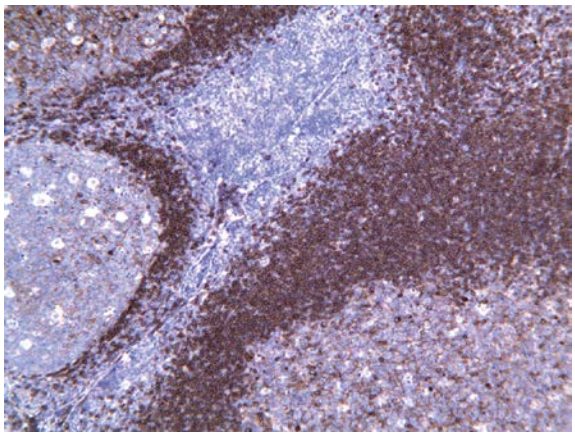

Fig. 19.14 Demonstration of IgM in a formalin-fixed paraffin-embedded section of reactive tonsil, using a labeled streptavidin-biotin immunoperoxidase technique with DAB as the chromogen.

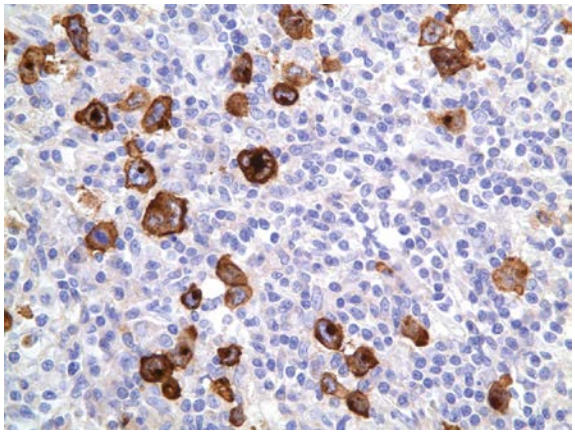

Fig. 19.15 CD30 demonstration in a formalin-fixed paraffin-embedded section of Hodgkin's disease. Note the strong staining of both the membrane and Golgi apparatus.

often then be applied. Referral laboratories dealing with a wide range of material from different sources where the fixation regimes are unknown may need to use more than one retrieval method.

The advances in molecular technology enabled the production of duplicate synthetic peptide sequences which survive routine processing techniques. One such peptide was produced for the human CD3 antigen, fixed in a formalin fixative and subsequently used to raise a polyclonal antibody (Mason et al., 1989). This reagent proved to be successful in the detection of T cells and related lymphomas in routinely processed paraffin wax sections. Using either proteolytic digestion or heat antigen retrieval, polyclonal anti-CD3 is an excellent example of how IHC has adapted to the prevailing conditions in histopathology.

Frozen sections

Despite the decline in the use of frozen sections for diagnostic purposes, IHC on frozen sections remains an important histological tool. Frozen sections have certain inherent disadvantages compared to paraffin wax sections, including poor morphology, limited retrospective studies and storage of material. The advantage of frozen sections is that the antigen is preserved and not cross-linked or hidden as can occur in paraffin wax sections.

The use of air-dried, unfixed cryostat sections of skin or renal biopsies allows the detection of chemically sensitive or otherwise labile antigens present in the tissue. In practice, unfixed cryostat sections or cell preparations are used whenever possible unless the antigen under investigation is known to be soluble, in which case suitable fixation such as cold 10% neutral buffered formalin can be used. Ethanol and acetone are other alternatives, but it should be noted that tissue morphology is sub-optimal with these methods.

Cytological preparations

The value of IHC on cytological preparations has been established. Acetone-fixed smears or cytospins are often preferred by the immunohistochemist, as this allows a wide range of primary antibodies to be employed without destroying the target epitopes. Many cytology laboratories still insist on fixing cytological preparations in alcohol as opposed to acetone, and consequently the number of demonstrable antigens may be limited, but the morphology may be superior. Where cytological samples are cellular enough, there is also the option of producing a cell block which can then be processed into paraffin wax. This gives greater flexibility for the number and range of tests which can then be performed on the sample and also provides archive material.

Automation

Following the introduction of heat antigen retrieval methods, there has been a continual increase in the numbers of antibodies effective on paraffin wax sections. Many of these antibodies can have a direct impact on diagnosis, prognosis or treatment. The move towards automation has also been driven by other forces, such as the need for greater standardization and consistency of results, a change in workforce, and the need to comply with national accreditation standards. Reflecting the current diagnostic turnaround targets there is now constant pressure to get a diagnostic result back to the clinician in as short a timeframe as possible. Many large diagnostic laboratories can be processing between 50-80,000 IHC slides per year. The use of automated systems not only increases productivity but also enhances quality assurance with full traceability of reagents. This is becoming more important for accreditation standards. Alongside automated staining systems many laboratories have also taken the step to move to pre-diluted antibodies. They are generally of good quality and produce consistent, reliable staining, as well as saving time and reducing errors e.g. less pipetting.

The selection of a suitable autostainer may be influenced by many factors but should take into account aspects such as:

- Capacity
- Flexibility
- Reliability
- Ease of use
- Cost per slide.

The more recently introduced autostainers, e.g. the Dako OMNIS and Ventana Benchmark Ultra, allow a LEAN workflow pattern, moving away from batch processing to a more continual loading of slides. This allows greater flexibility and makes prioritization of cases much easier.

Wherever possible, it is recommended to trial the different autostainer options, and assess them with the laboratory's own material and their routine antigen panels. Prioritization of the automated immunostainer criteria will differ between diagnostic and research facilities. The options have previously been outright purchase, lease of the equipment and/or a reagent rental contract. Most diagnostic laboratories would normally go with a reagent rental contract now, particularly as they may need multiple instruments to fulfill their capacity requirements. Each method has its advantages and disadvantages. Purchase involves one payment but does not include maintenance after the initial warranty or replacement costs. Leasing of the equipment or a reagent rental contract will cover the maintenance costs and equipment is replaced as required.

Automated incubation methods

Automated IHC systems such as the Leica Bond III use a flatbed system with unique Covertile™ technology facilitating uniform staining of the tissue. Reagent is gently applied at one end of the covertile and gently flows along under the covertile to fully cover the section and prevent drying out. The full IHC procedure, including dewaxing and antigen retrieval, is carried out using this automation. The Dako OMNIS autostainer has on board dewaxing and antigen retrieval facilities, and uses capillary gap technology in a heated staining module for the incubation and washing stages. The Ventana Benchmark Ultra uses a kinetic mode system involving air-vortex mixers to mix the reagents, liquid coverslips to prevent drying out, and a thermoflex pad to provide precise heating across the whole slide. Dewaxing and antigen retrieval is carried out on-board. These are just three examples of automated IHC staining systems, others exist and all work well.

Blocking endogenous enzymes

If endogenous enzymes similar to those used as the antibody label are present in the tissue, they may react with the substrate used to localize the tracer and give rise to problems in interpretation. Inhibiting endogenous enzyme activity prior to staining can eliminate false positive reactions produced in this way. Peroxidase and substances

giving a pseudoperoxidase reaction are present in some normal and neoplastic tissues, e.g. leukocytes and erythrocytes, and various methods have been described for the destruction of their activity. The most frequently used method is pre-incubation of the sections in absolute methanol containing hydrogen peroxide (Streefkerk, 1972). Incubation in absolute methanol containing 0.5% hydrogen peroxide for 10 minutes at room temperature has been reported to produce an almost complete abolition of endogenous peroxidase activity, without affecting the immunoreactivity of antigens (Delellis et al., 1979). The mechanisms of inhibition and details of other methods have been reviewed by Straus (1976).

There are many types of alkaline phosphatase within the human body, and most endogenous alkaline phosphatase activity can be blocked using a 1 mM concentration of levamisole in the final incubating medium. The alkaline phosphatase used in the labeling system is usually intestinal in nature and remains unaffected by levamisole at the recommended concentration. Using 20% acetic acid can block intestinal alkaline phosphatase, but the acidic treatment may damage some antigens.

Other enzyme labels such as glucose oxidase and bacterial β-*D*-galactosidase, do not present a problem. The former does not have active endogenous enzyme in mammalian tissue, the latter does, but the label and chromogen react at a different pH from the mammalian enzyme.

Blocking background staining

The major causes of background staining in IHC are hydrophobic and ionic interactions and endogenous enzyme activity. Background staining may be specific e.g. fibrinogen in blood vessels and immunoglobulins in serum-bearing tissues, or non-specific due to the apparent affinity of certain tissue components. Non-specific uptake of antigen, particularly the high affinity of collagen and reticulin for immunoglobulins, can cause high levels of background staining.

Hydrophobic interactions are the result of the cross-linking of amino acids, both within and between adjacent protein molecules. Proteins are rendered more hydrophobic by aldehyde fixation and the extent of hydrophobic cross-linking of tissue proteins is primarily a function of fixation.

Tissues which give background staining as a result of hydrophobic interactions include collagen and other connective tissues, epithelium and adipocytes (Kraehenbuhl & Jamieson, 1974). Hydrophobic bonding can be minimized by the addition of a blocking protein, a detergent such as Triton X (Hartman, 1973) or a high salt concentration (2.5% NaCl) to the buffer (Grabe, 1980). Some workers advocate the addition of the blocking serum to the diluted primary antibody (Delellis et al., 1979).

Non-specific staining is most commonly produced because the primary antibody is attracted non-immunologically to highly charged groups present on connective tissue elements. Positive staining is not due to localization of the antigen but to non-specific attachment of the primary antibody to connective tissues. Since the primary antibody is attached to connective tissue moieties, the subsequent labeling antibodies will be attracted to primary antibodies located on the specific antigen as well as the antibody bound to the connective tissue elements.

The most effective way of minimizing non-specific staining is to add an innocuous protein solution to the section before applying the primary antibody. The added protein should saturate and neutralize the charged sites, enabling the primary antibody to bind only to the antigenic site.

Traditionally, non-immune serum from the animal species in which the second (bridging) antibody was raised is used as a blocking serum. In practice any animal serum or protein, e.g. casein, can be used for this purpose as long as the protein used as a block cannot be recognized by any of the subsequent antibodies used in the technique.

In frozen sections and cytological preparations, tissue receptors for the Fc portion of antibodies may give rise to additional problems. Fc receptors are present on several cell types such as macrophages and monocytes, and are largely destroyed by formalin fixation and paraffin wax processing. If necessary Fab fragments of antibodies which lack the Fc portion should be used.

Several authors also found that enzymatic digestion reduces non-specific background staining (Huang et al., 1976; Curran & Gregory, 1977; Denk et al., 1977).

In immunofluorescent techniques high background, or false positive, staining may occur because of inadequate washing of the slides following antibody or conjugate incubation. It may also occur if the sections have been allowed to dry out during incubation. High background staining may also be due to a high fluorochrome to protein ratio, from free chromophore in the conjugate, or over-incubation of the tissue with reagent.

Controls

Controls validate IHC results and are an essential part of the staining process.

Many laboratories are now running a control on the same slide as the test section as this gives better validation of the result. The use and type of controls are discussed in much greater detail in the quality control section later in this chapter.

Negative control

The production of a negative control involves either the omission of the primary antibody from the staining schedule or the replacement of the specific primary antibody by an immunoglobulin which is directed against an unrelated antigen. This immunoglobulin must be of the same class, source and species. A number of negative control cases should be tested as part of an antibody validation, verifying that it will not produce false positive staining of other tissue constituents.

Positive control

The absence of staining in a test section does not necessarily imply that the antigen is not present – the use of a section of known positivity is always advisable. For validation purposes it is important to test a number and range of positive control cases. This will help to ensure that the antibody is suitable for the demonstration of both low and high expression of proteins. Positive elements within test sections, e.g. normal reactive lymphocytes when staining with an antibody to the leukocyte common antigen used to identify a suspected lymphoma, are a useful adjunct, but should not replace a true positive control.

Every batch of slides prepared for immunofluorescence staining should include slides with known antigen deposits as quality control slides. Microscopic examination of these slides should include an evaluation of the fluorescence intensity and amount of background staining compared to previous results, as well as the identification and location of the deposits and the integrity of the tissue. The batch should be rejected if the quality control slide results are aberrant. Negative slides, i.e. those without antibody, can also be included to indicate non-specific binding.

Absorption control

The ideal negative control demonstrates that immunoreactivity is abolished by pre-absorption of the specific primary antibody with the purified antigen. If staining does occur after absorption then the staining must be due to a contaminating antibody and not to the antigen-antibody interaction under investigation. This type of absorption control is necessary in the characterization and evaluation of new antibodies. This may be regarded as the ultimate test for specificity (Fig. 19.16). It is rarely used in diagnostic work as well-characterized commercial antibodies are available. An additional useful control is to block the binding between the primary antibody and conjugated antibody in the traditional indirect method. This is achieved by interposing an incubation in unlabeled immunoglobulin of the type present in the labeled antibody between incubations of the two relevant antibodies.

Practical aspects of immunohistochemical staining

The practical aspects of IHC staining are simple and straightforward, as the techniques entail only sequential incubations in antibodies and labeling systems separated by washes in buffer. To obtain optimal staining and prevent the non-specific precipitation of antibody onto the sections, it is essential to ensure that each antibody is used at an

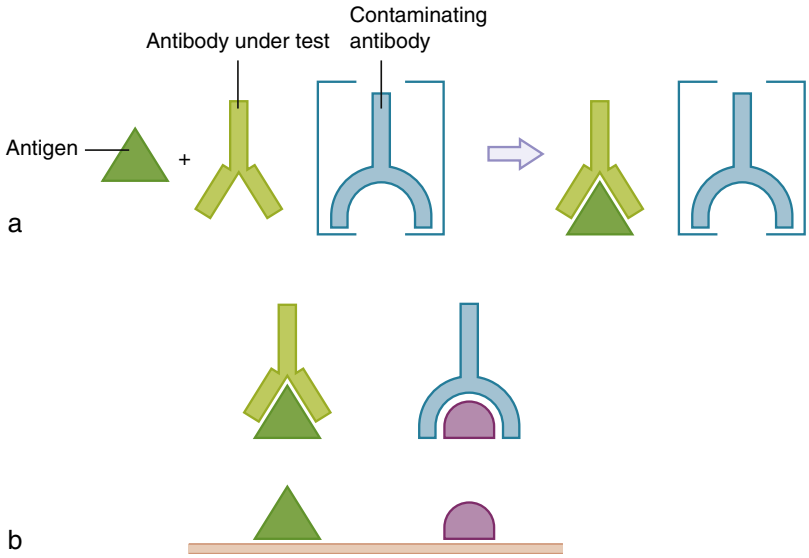

Fig. 19.16 Absorption control. (a) The antibody under test is reacted with its antigen to give an immune complex reaction with no staining by the contaminating antibody. (b) This test section shows the antibody reacting with the target antigen and the contaminating antibody binding to an alternative antigen.

appropriate dilution, does not evaporate during incubation and is completely removed, if unbound, before the next specific antibody or reagent is added.

Dilution of immune serum/antibodies

For optimal staining to occur it is necessary to use the primary specific antibody at the correct dilution. Incorrect dilutions can give rise to false negative results, particularly in antigen-rich tissues (Bigbee et al., 1977).

When applying an untested antibody to a tissue section containing the relevant antigen, a broad dilution series should be used to ensure that false negative results do not occur. Selection of the cleanest dilution with an intense signal, particularly on normal cells, is not always the optimal dilution for diagnostic material. Tumor cells do not always have the full compliment of antigens seen in the background tissues. Dilutions which identify normal cells may be too dilute to demonstrate the tumor cells. It is recommended that dilution factors are not set at the extreme end of the range and, where possible, it is useful to test out dilution factors on known neoplastic material if appropriate.

Theoretically, in multi-layer techniques each separate stage should be titrated against the other antibody stages and the optimal concentration selected for each antibody. In practice, most commercially supplied primary antibodies and labeling systems are provided with a recommended dilution range. It is usually only the primary antibody dilution which needs to be adjusted.

Washes

To prevent the formation of antigen-antibody complexes which will precipitate onto the sections and give rise to problems with interpretation and background staining, it is necessary to remove the unbound antibody before incubation in the next layer. This is achieved by washing the sections between antibody incubations in Tris-buffered saline (TBS). This solution may be made up in bulk for convenience.

For routine work, a few brief washes with TBS will usually suffice. In the past some workers recommended the addition of detergent such as BRIJ 96 (Sigma) to the washing solutions (Heyderman & Monaghan, 1979). Today the most popular surfactant is Tween 20, often used in concentrations of 0.01–0.05%.

Buffer solutions

0.05 M Tris-buffered saline (TBS)

Distilled water	10 L
Sodium chloride	85 g
Tris (hydroxymethyl) aminomethane	60.5 g

Adjust pH to 7.6 with concentrated hydrochloric acid.

Tris-buffered saline containing bovine serum albumin (BSA-TBS)

Tris (hydroxymethyl) aminomethane	12.14 g
Sodium chloride	45 g
Bovine serum albumin	5 g
Sodium azide	6.5 g
Distilled water	5 L

Adjust final pH to 8.2 with 1 M hydrochloric acid.

Heat-mediated antigen retrieval fluids

Tri-sodium citrate buffer

Tri-sodium citrate	19.7g
Distilled water	5 L

Adjust to pH 6.0 with 1M Hydrochloric acid

Tris-EDTA

Tris	14.4 g
EDTA	1.44 g
1 M hydrochloric acid	1 ml
Tween 20	0.3 ml
Distilled water	600 ml

Add the Tris, EDTA, and acid to the distilled water and adjust pH to 10 with 1M hydrochloric acid, then add the Tween.

Manual incubation methods

To prevent evaporation of antibodies, incubations must be carried out in a moist atmosphere. This is most easily obtained by placing the slides on Perspex strips which run the length of a lidded staining trough which has, a pool of water or a layer of moist tissue paper at the bottom, the incubation chamber.

It is advisable to leave a small gap between adjacent slides so that cross-contamination of antibodies cannot occur. Using moist incubation chambers, it is not necessary to flood the slide with antiserum. If the area around the section is dried thoroughly with a tissue before application of the antibodies and labeling systems, a few drops of reagent should suffice. Additionally the use of a wax pen around the sections assists with retaining the reagents on the sections. Other methods beyond routine techniques are occasionally needed, usually in research or specialist laboratories. One such example is the demonstration of hormones and neuropeptides which require special fixation (Van Noorden et al., 1986). To demonstrate these hormones, the use of freeze-dried material which is vapor fixed in *p*-benzoquinone or diethyl pyrocarbonate, and subsequently paraffin wax embedded, is recommended (Pearse & Polak, 1975).

Method selection

The majority of immunohistochemists in the UK using automation use a polymer chain two-step indirect method for routine work. The peroxidase-DAB reaction end product is also preferred, because it is resistant to processing to resinous mounting media and has long-term storage qualities. The storage qualities are particularly important when diagnoses are under review over prolonged periods of time.

Preparative technique

Preparing paraffin wax sections for manual immunostaining

To prevent section damage or detachment the sections should be picked up on charged slides or on slides coated with a strong adhesive such as Vectabond (Vector Laboratories) or amino-propyltriethoxysilane (APES). Sections should be cut at 3-4 µm.

1. Dry the sections overnight in a 37°C incubator. Alternatively, the sections may be placed in a hot oven at 60°C for approximately 30 mins. Hot plates are not recommended as certain antigens, such as estrogen and progesterone receptors, show a reduction in staining when heated on a hot plate.
2. Where possible, sections should be cut fresh. Sections stored for several weeks prior to immunostaining may show reduced staining intensity; this is the case with estrogen and progesterone receptors.
3. Dewax sections in xylene and take to absolute alcohol. Xylene substitutes such as Histoclear may be used. To ensure complete removal of wax the xylene/Histoclear may be warmed to 37°C in an incubator.

4. If required, remove fixation pigments.
5. Block endogenous peroxidase activity by incubating in 0.5% hydrogen peroxide in methanol for 10 minutes. This stage may be performed after the primary antibody has been bound onto the antigenic site. It is thought by many users that the methanol/hydrogen peroxide step may slightly alter some of the more labile antigenic epitopes, leading to weak demonstration. We have certainly found this to be the case with the demonstration of CD2 and CD4 in paraffin wax sections.
6. Rehydrate, wash well in running water.
7. Perform the required/preferred antigen retrieval techniques as detailed below.

Antigen retrieval techniques

Proteolytic enzyme methods

The digestion media outlined below must be freshly prepared as their activity decreases with time. Digestion methods with proteolytic enzymes are usually limited to sections taken from formalin-fixed paraffin-embedded tissues.

Optimal times for digestion are dependent on fixation parameters. These vary according to specimen size, temperature of fixative, duration of fixation and the rate of penetration of the fixative. With such variables a fixed digestion time does not always achieve optimal staining. Usually a uniform time, established on controls, is used in the initial stages. Antibodies such as those for pan-cytokeratin will react to a satisfactory standard even when the digestion time is suboptimal, but others such as immunoglobulin light chains require a more precise methodology.

The following methods should be used as a guideline only. We would recommend that individual laboratories determine individual digestion times on their own material.

Trypsin/chymotrypsin

Method

1. Incubate sections in distilled water at 37°C.
2. Prepare 0.1% trypsin in 0.1% calcium chloride in distilled water at 37°C. Adjust pH to 7.8 using 0.1 M sodium hydroxide solution.
3. Incubate the sections in the trypsin solution for 10 minutes at 37°C.
4. Wash sections in cold running tap water to prevent further digestion.
5. Proceed with the immunostaining method of choice.

Note

A successful alternative to trypsin is chymotrypsin (Sigma C-4129) (Miller et al., 1995b).

Protease

Method

1. Incubate sections in pre-warmed distilled water at 37°C.
2. Prepare 0.1% protease (Sigma type XXIV, P-8038) in distilled water (at 37°C). Adjust pH to 7.8 using 1 M sodium hydroxide solution.
3. Incubate the sections in the protease solution for 6 minutes at 37°C.
4. Wash sections in cold running tap water to prevent further digestion.
5. Proceed with the immunostaining method of choice.

Note

Using 0.05% protease may be preferred. By using a less concentrated solution the possibility of over-digesting the tissue sections is decreased. Digestion times will require increasing but are not as critical as with the 0.1% protease solution.

Pepsin

Method

1. Incubate sections in pre-warmed distilled water at 37°C.
2. Prepare 0.4% pepsin solution in 0.01 M hydrochloric acid, pH 2.0, at 37°C.
3. Incubate the sections in the pepsin solution for 15–60 minutes at 37°C.
4. Wash sections in cold running tap water to prevent further digestion.
5. Proceed with the immunostaining method of choice.

Note

Certain antigens, e.g. basement membrane proteins, give improved staining if the digestion is performed by pepsin.

Heat-mediated antigen retrieval

There are now many heat retrieval methods employing different types of equipment and various retrieval solutions. If not using automation then the equipment required includes a microwave oven, pressure cooker, steamer and/or water bath. Among the various solutions, tri-sodium citrate buffer at pH 6.0, EDTA at pH 8.0, and Tris-EDTA (pH 9.9 or 10.0) are the most popular. Laboratories using automated systems with their own commercial reagents usually have either a high pH or a lower pH 6.0 option.

Microwave oven heating methodology

Jessup (1994) reported that it is possible to achieve relatively even exposure of antigen across the tissue section by heating 10 slides in a plastic slide rack in a deep microwavable container (e.g. Addis 9400) holding 600 ml of fluid. This method has since been developed and a maximum of 25 sections can be heated per batch. With this type of tall narrow container, the height of the fluid above the slides removes the need for topping up as there is no risk of the slides drying out over a 30-minute heating cycle. A loose lid is required to reduce the volume of fluid lost, but allows the steam to escape.

Method

1. Using a plastic staining rack, place up to 25 sections in 600 ml of 0.01 M citrate buffer, pH 6.0.
2. Irradiate at a power of 800 W for 22 minutes.
3. Carefully remove the container from the microwave oven and flood with cold water.
4. Proceed with the immunostaining method of choice.

Note

For suboptimally fixed material we recommend reducing the volume of buffer to 400 ml and the heating time to 15 minutes.

Pressure cooker antigen retrieval methodology

Use a 5 liter domestic stainless steel pressure cooker with an operating pressure of 103.4 kPa. To bring the pressure cooker to boil use either a halogen hot plate or domestic electric hot plate. A maximum of three racks of 25 slides each can be pretreated at one time.

Method

1. Add 1.5 L of appropriate antigen retrieval buffer into the pressure cooker and bring to the boil without securing the lid.
2. When the antigen retrieval buffer is boiling, carefully place the slide racks into the hot solution and seal the lid.
3. Allow the pressure cooker to reach full pressure 10.3 kPa (15 psi), incubate for 2 minutes when full pressure is reached.
4. Transfer the pressure cooker to a sink and run cold water over the lid until all of the pressure is released.
5. Flood the pressure cooker with cold water. Do not remove the slides until cool.
6. Proceed with the immunostaining method of choice.

Note

Plastic pressure cookers are now available for heating in a microwave oven, but they have a lower operating pressure than the stainless steel versions and the heating time at full pressure has to be significantly increased.

Steamer antigen retrieval methodology

This method uses a domestic rice steamer.

1. Place 1 L of distilled water into the reservoir in the base of the steamer.
2. Place a steaming tray onto the base.
3. Place the rice bowl into the steaming chamber.
4. Fill a plastic Coplin jar/slide holder with the appropriate antigen retrieval buffer and place in the rice bowl.
5. Place the lid onto the top of the steaming chamber.
6. Set the timer for 1 hour 15 minutes. The equilibration of the bath/rice chamber contents to 95°C is achieved after around 45 minutes.
7. Remove the lid and place the slides in the heated antigen retrieval buffer; replace the lid.
8. Incubate sections for 30 minutes.
9. Remove the slide container from the rice bowl and allow the sections to cool for 15 minutes.
10. Wash sections in water.
11. Proceed with the immunostaining method of choice.

Water bath antigen retrieval methodology

This method uses a conventional laboratory water bath.

1. Place the appropriate antigen retrieval buffer in a plastic Coplin jar.
2. Place the Coplin jar into the water bath and heat to 95–98°C, without boiling.
3. Place slides into the preheated buffer and incubate for 30 minutes.
4. Remove the container from the water bath and allow cooling for 15 minutes at room temperature.
5. Wash sections in water.
6. Proceed with the immunostaining method of choice.

Examples of immunostaining protocols for routine diagnostic antigens

Testing all the clones available for a particular epitope would be too costly. Most manufacturers and suppliers provide a data sheet with each of their antibodies, which will give recommendations as to their use. If a more independent guide is sought, then *Leong's Manual of Diagnostic Antibodies for Immunohistology* (Chetty et al., 2016) is recommended.

Immunohistochemistry for immunoglobulin light chains in formalin-fixed paraffin-wax sections

This is one of the most difficult of all techniques to perform. The retention of the immunoglobulin in the appropriate cells with good fixation is a prerequisite of producing good light chain demonstration. Furthermore, light chain restriction is an important criterion when identifying B-cell lymphoma and this restriction is one of few markers in this malignancy available to immunohistochemists today. Prompt fixation in either a buffered or unbuffered 10% formalin solution is essential. Lymph nodes and other dense lymphoid tissue should be sliced as soon as possible in order to facilitate the penetration of the fixative. Ideally, fixation for 18–72 hours is acceptable, but once fixation time is extended to several weeks, the demonstration of light chain immunoglobulin becomes increasingly difficult.

The accuracy of staining has been verified by comparing IHC results on sections with flow cytometric analysis of fresh lymphoid tissue. Flow cytometric analysis requires fresh tissue to be perfused with an isotonic solution and any red cell contamination to be lysed with ammonium chloride. The lymphocytes are labeled with a fluorescently conjugated primary antibody, washed with an isotonic solution and analyzed on a flow cytometer. Flow cytometric analysis is the optimum method for immunophenotyping lymphomas on fresh tissue but many laboratories do not have the resources to undertake this and many lymphomas present as formalin-fixed, paraffin wax-embedded blocks.

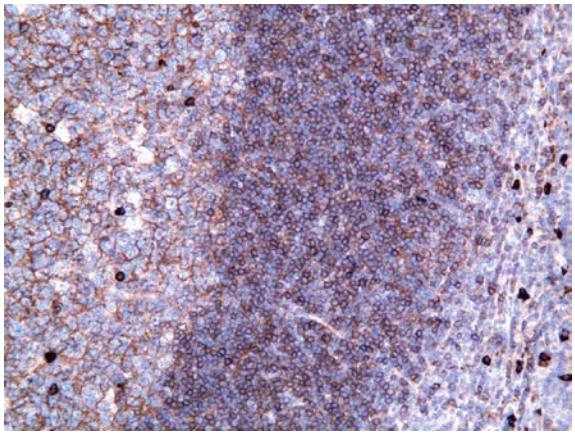

Fig. 19.17 Formalin-fixed paraffin-embedded section of reactive tonsil demonstrating kappa light chain immunoglobulins. Strong staining of plasma cells, follicular dendritic cells and mantle zone B cells are clearly visible. Antigen retrieval using the microwave oven and citrate buffer, pH 6.0.

Sections of reactive tonsil provide the ideal control material for light chain demonstration. Tonsil sections should show marked demonstration of the mantle zone and follicle center B cells, together with intense plasma cell staining (Fig. 19.17). The T-cell zone should have little staining. Background immunoglobulin, caused essentially by non-specific uptake, is seen in follicular dendritic cells, some connective tissue and epithelia.

If there is any doubt, it is important to establish on the test material that small round B cells (see Appendix III) are present with a series of B markers. This will usually assist with identifying B cells at different maturation stages. Once achieved, immunoglobulin staining can be compared with the control section. If there are only a few plasma cells and little

else staining, then it is almost certain that the antigen retrieval has been underachieved. Increasing the antigen retrieval times on duplicate sections will increase the reactivity, and perinuclear space and surface staining of the light chains on the B cells will indicate optimal retrieval. In addition, intense cytoplasmic staining of immunoglobulin in plasma cells should be seen. Excessive antigen retrieval, by heat or by proteolytic digestion, will cause staining of large amounts of reticulin, and could remove some of the cells and protein structures from the slide.

Reliable light chain demonstration on paraffin wax sections can be achieved using either two-step indirect polymer-labeled methods with DAB as the preferred chromogen, or by polymerase chain-based techniques. If a manual antigen retrieval is used, we would recommend antigen retrieval using microwave oven heating with citrate buffer but optimum results are seen using automated IHC platforms.

The choice of antigen retrieval will depend upon the individual laboratory and their fixation and processing regimes. Polyclonal primary antibodies are recommended.

Immunohistochemistry for the assessment of HER2 expression

The assessment of HER2 in human tumor cells has recently become important since the amplified gene is predictive of response to the novel humanized HER2 antibody, Herceptin®/trastuzumab. Historically the major factors directing the appropriate treatment for breast cancer have been those regarding prognosis, i.e. indicators of how long the average patient is likely to survive. As a result, the relative aggressiveness of a tumor has been gauged and the appropriate treatment regimen has been planned. Recently the concept of predictive factors has risen to the fore. Unlike prognostic factors, which are concerned with the likelihood of a particular outcome, the predictive factors are concerned with identifying features of a tumor which will direct specific targeted therapies or an improved outcome with combinations of chemotherapeutic agents.

HER2 is a member of the epidermal growth factor receptor (EGFR) family of molecules and is encoded for by the HER2 proto-oncogene on the long arm of chromosome 17. HER2 is overexpressed in 10–20% of primary breast cancers, indicating a poor prognosis. These cancers are candidates for treatment with trastuzumab. Studies show that trastuzumab can significantly reduce the risk of reccurrence and mortality in these early-stage breast cancer patients.

Formalin-fixed paraffin wax sections immunostained to demonstrate HER2 are examined microscopically and scored. Only invasive breast cancer cells should be scored. Non-invasive tumors i.e. ductal carcinoma in situ, are not assessed. If an automated system is being used it is likely that a validated kit is used to perform the test, and the laboratory should follow the manufacturer's recommended protocol for staining. Where this is not an option and manual staining is performed it is recommended that formalin-fixed paraffin wax sections should be antigen retrieved using the water bath antigen retrieval method and citrate buffer, pH 6.0, to ensure optimal immunostaining.

When assessing HER2 staining, the following scoring system is used (Fig. 19.18):

0 No staining at all or very slight partial membrane staining in less than 10% of tumor cells.

1+ Faint barely perceptible membrane staining in more than 10% of tumor cells. Cells stained in only part of the membrane.

2+ Weak to moderate complete membrane staining observed in more than 10% of tumor cells.

3+ Strong complete membrane staining in more than 30% of tumor cells.

Application of fluorescence in situ hybridization (FISH) for HER2 assessment (Fig. 19.19)

The following is the current practice in the UK:

- The centromeric region of chromosome 17 is marked with a green fluorescent signal and the HER2 gene with a red signal.
- Assess 20–60 cells: count the number of red: green signals in each cell.
- The overall gene-to-chromosome ratio is calculated.

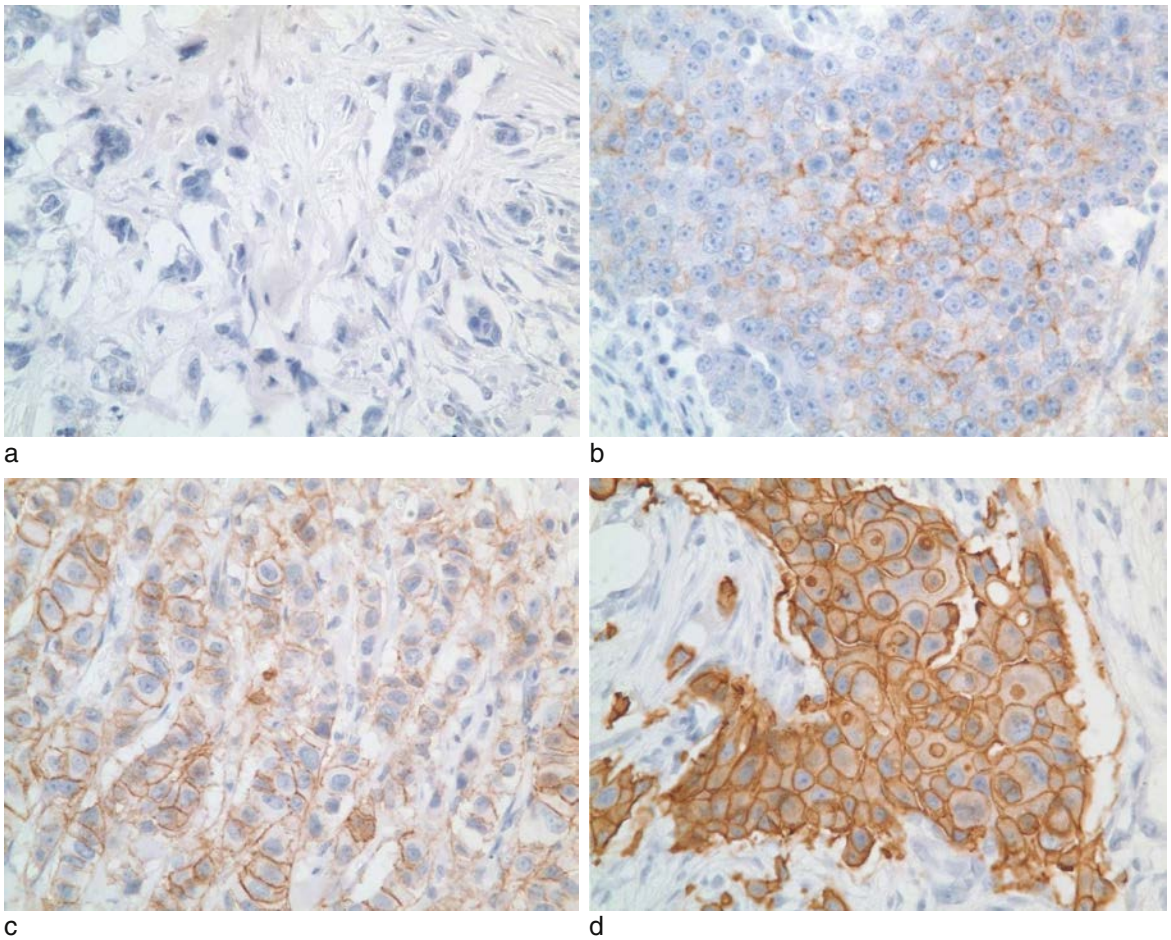

Fig. 19.18 Breast tumor HER2 assessment grading: (**a**) 0, (**b**) 1+, (**c**) 2+, (**d**) 3+.

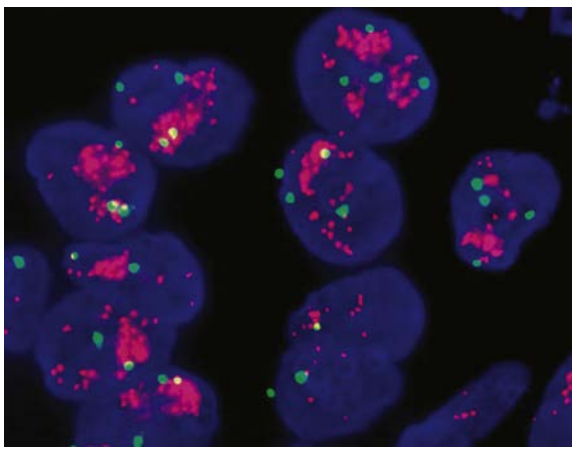

Fig. 19.19 FISH HER2 demonstration, showing amplification of the HER2 gene (red fluorescence).

- A tumor is designated *positive* if gene-to-chromosome ratio is >2.2 and *negative* if <1.8. For gene counts which fall in between 1.8 and 2.2 we class as *equivocal* then assess more cells and make a call in conjunction with the IHC.

Application of chromogenic in situ hybridization (CISH) as an alternative to FISH (Figs. 19.20 and 19.21)

CISH is:

- A similar technique to FISH but allows the chromogenic visualization of the HER2 gene and/or chromosome 17 on either the same or separate slides.
- Often reported as either a direct count if signal quality allows or may be reported as signal clusters.

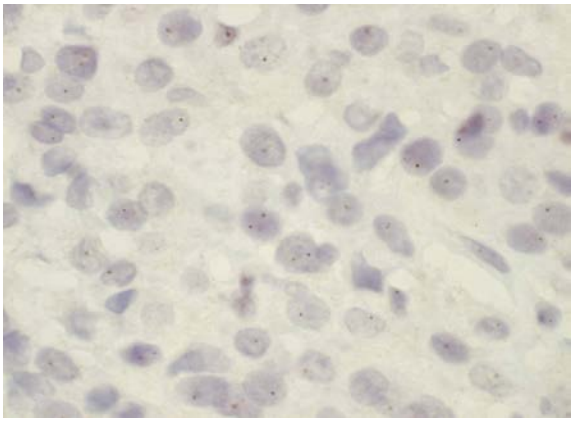

Fig. 19.20 CISH HER2 demonstration, showing single signal count.

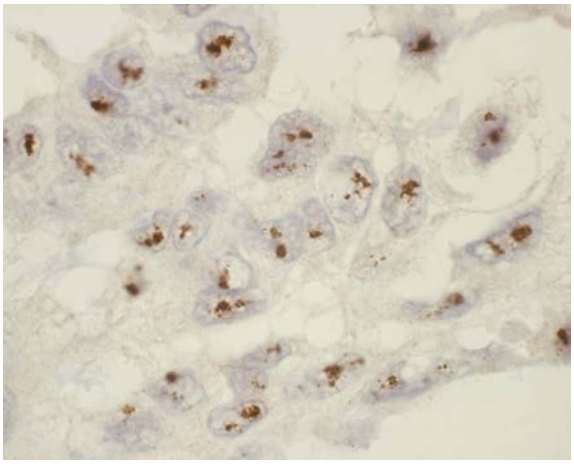

Fig. 19.21 CISH HER2 demonstration showing signal clusters.

Immunohistochemistry on frozen section and non-gynecological cytology smears

Cytological preparations, e.g. smears, imprints and cytospins should be air dried for 1–3 hours and then fixed or stored as outlined in the method below for frozen sections.

Method for frozen sections

1. Cut 6 µm frozen sections and place on Superfrost Plus microscope slides or adhesive-coated slides.
2. Air dry the sections at room temperature overnight or in urgent cases dry for a minimum of 1–2 hours.
3. Fix sections in absolute acetone at room temperature for 20 minutes. Allow sections to air dry. If required, the sections may be stored at this stage at –20°C or lower. Prior to storage, the slides should be wrapped in foil and then placed in the freezer with a desiccant. When required, the sections should be allowed to return to room temperature before unwrapping.
4. Rehydrate in TBS, apply optimally diluted primary antibody. Antibodies should be diluted in TBS avoiding the use of commercial antibody diluents or the use of detergents such as Triton or Tween 20. Chromatolysis and loss of nuclear membranes on frozen sections is compounded by the action of detergents.

Note

For frozen sections an endogenous peroxidase-blocking step is not included, as this can be damaging to the antigens to be demonstrated. The use of a negative control for the identification of endogenous peroxidase activity is preferable. If the endogenous peroxidase activity is excessive, then an alternative enzyme tracer, such as alkaline phosphatase, should be considered.

Immunohistochemistry on renal and skin biopsies

Whilst the frozen fluorescence technique requires a high degree of skill, there is no doubt that the paraffin wax method provides a difficult technical challenge. Furthermore, such is the capriciousness of this method that maintaining the standards of an established immunoperoxidase technique for these purposes requires a dedicated and highly skilled immunohistochemist. There are some important reasons why opinion is divided as to the preferred technique. McIver and Mepham (1982) reported that immunoperoxidase was their established method for demonstrating immunocomplexes in renal biopsies. Turbitt et al. (1982) also described an immunoperoxidase method to demonstrate autoantibodies in the bullous lesions of pemphigoid and pemphigus. Today, even with the disadvantage of having to split the biopsy for frozen, paraffin wax processing and electron microscopy, the method of choice for many is still frozen section immunofluorescence coupled with paraffin wax section morphology. The advantages and disadvantages of immunofluorescence and immunoperoxidase are outlined in the Tables 19.2 and 19.3.

Table 19.2 Advantages of immunofluorescence and immunoperoxidase

Frozen section immunofluorescence	Paraffin wax section immunoperoxidase
Usually involves a simple, rapid, and sensitive direct technique.	All or a substantial part of the biopsy (except for renals where a small portion is processed for electron microscopy) is formalin fixed and paraffin wax processed.
Easily reproduced.	Immunolocalization and morphology are clearly seen in the same section.
Histopathologists experienced with fluorescent antibody techniques in renal and skin biopsies find little difficulty with interpretation.	Peroxidase has good long term storage qualities, especially if diaminobenzidine chromogen (DAB) is employed.
	An expensive fluorescence microscope is not required. Processing, section cutting and storage of blocks are compatible with a routine diagnostic service.

Table 19.3 Disadvantages of immunofluorescence and immunoperoxidase

Frozen section immunofluorescence	Paraffin wax section immunoperoxidase
The production of good quality frozen sections from small biopsies requires a high degree of skill.	A more time-consuming, sensitive technique, employing proteolytic enzyme antigen retrieval, is required.
A fluorescence microscope is required.	Proteolytic digestion must be tailored to fixation time.
Immunofluorescent labeling has poor storage qualities, and can often fade within days of the sections being immunostained.	The technique requires a high degree of skill to insure a reliable level of reproducibility.
The morphology of the tissue is not easily seen.	DAB (diaminobenzidine chromogen) is hazardous.
In addition to a formalin-fixed specimen, further tissue is required which may involve extra needle core passes and creates an increased clinical risk to the patient (Mölne et al., 2005)	ABC techniques can lead to nonspecific staining in renal tubules (Mount & Cooper, 2001)

Certain skin diseases have characteristic patterns of immunoglobulin deposition, usually in the upper dermis or at the dermo-epidermal junction. Immunofluorescent investigation is particularly useful in the diagnosis of bullous disorders, systemic lupus erythematosus (SLE) and the vasculitides. Some of the more common examples are summarized in Table 19.4 and discussed in more detail in Farmer and Hood (2000).

The immunofluorescence examination of frozen sections of renal tissue obtained by percutaneous needle biopsy or wedge biopsy allows the detection and distribution of antigens to be seen within the kidney. Different glomerular diseases show different and often specific patterns of distribution. Some of these patterns are illustrated in Figs. 19.22–19.26 and are discussed in detail in Jennette et al. (1998), Walker (2009), Satoskar et al. (2007) and Crosson (2007). Most of these investigations use direct immunofluorescence with FITC-labeled primary antibodies. Other antigens such as C4d deposition in transplant kidneys with suspected antibody-mediated rejection (Collins, 1999) require an indirect method.

Any fluorescent staining on the slide should be reported as antigen type (IgG, C3, κ, λ, CD3, etc.), intensity (+, ++, +++), location (mesangial, peripheral, basement membrane, etc.), pattern (linear, granular), and extent (focal, diffuse, global, segmental).

Table 19.4 Immunofluorescent patterns in selected skin diseases

Disease	Immune reactant	Location
Bullous pemphigus	IgG, C3 (linear chicken wire pattern)	Intradermal/desmosome
Bullous pemphigoid	IgG, C3 (linear pattern)	Basement membrane
Dermatitis herpetiformis	IgA, C3 (granular)	Basement membrane
Systemic lupus erythematosus (SLE)	IgG, IgA, IgM, C3 (granular)	Basement membrane
Bullous SLE	IgG, IgA, IgM, C3 (granular or linear)	Basement membrane
Linear IgA disease	IgA, C3 (linear)	Basement membrane

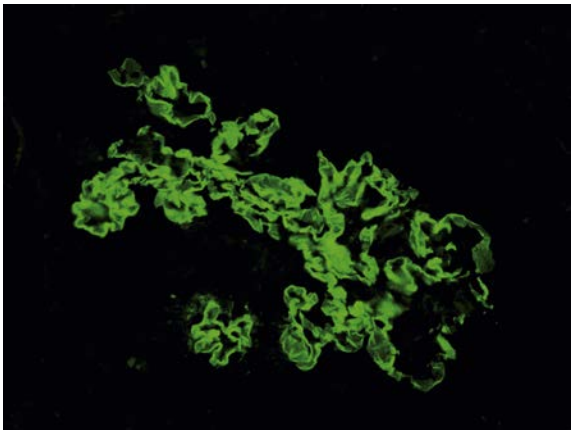

Fig. 19.22 Renal biopsy, immunofluorescent technique demonstrating glomerular basement membrane staining pattern, as seen in Goodpasture's syndrome.

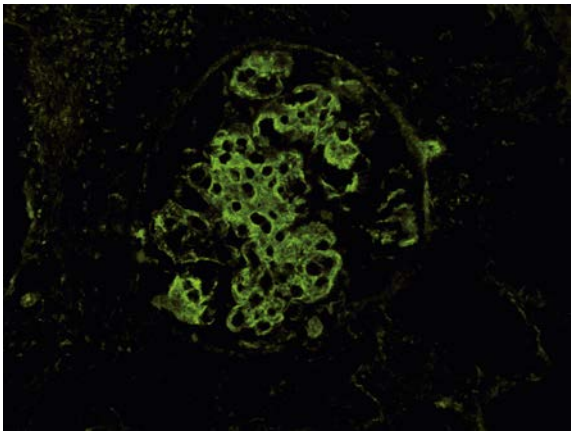

Fig. 19.24 Renal biopsy in SLE, immunofluorescent technique, demonstrating glomerular staining for C1q.

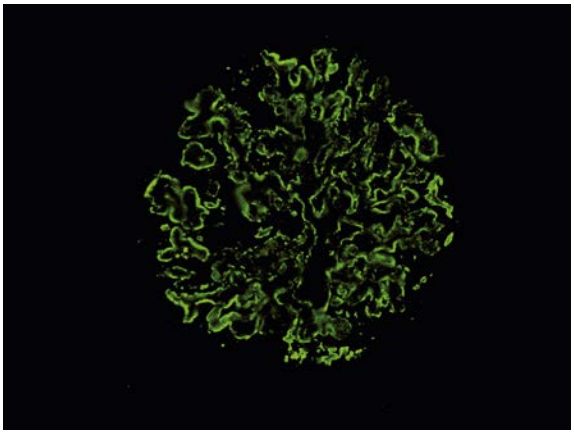

Fig. 19.23 Renal biopsy, immunofluorescent technique demonstrating membranous staining pattern with antibody to C3.

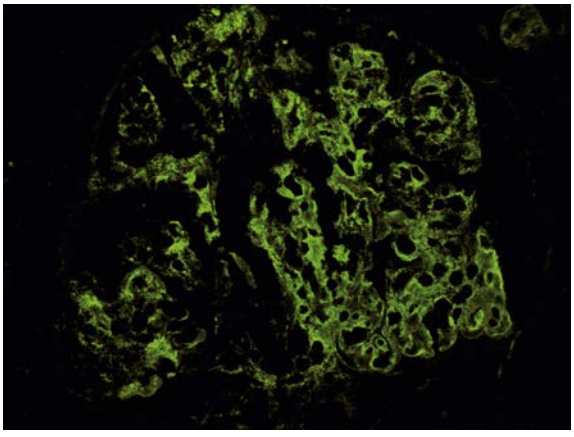

Fig. 19.25 Renal biopsy in SLE, immunofluorescent technique demonstrating glomerular staining for IgG.

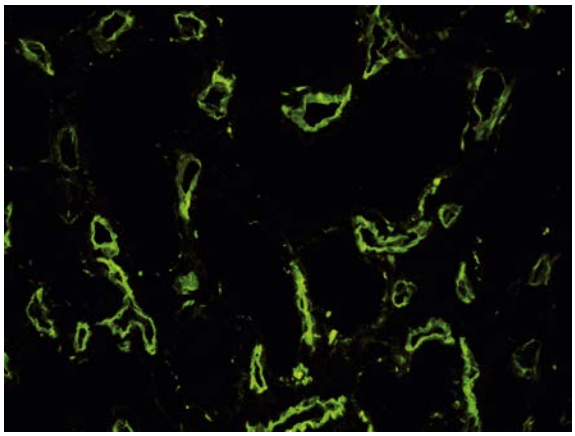

Fig. 19.26 Renal biopsy from a patient with kidney transplant rejection, immunofluorescent technique demonstrating staining of peritubular capillaries for C4d.

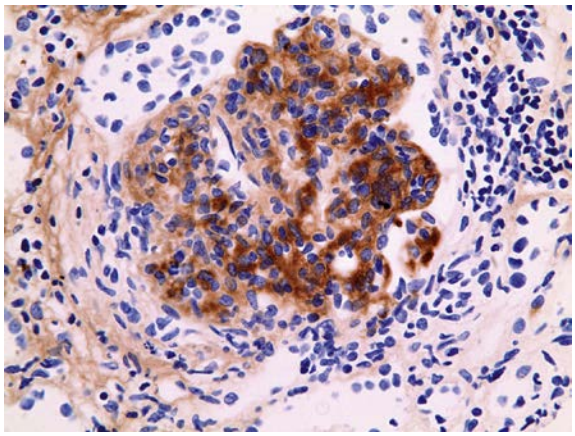

Fig. 19.27 Formalin-fixed paraffin wax-processed section of renal biopsy with IgA nephropathy, immunostained for IgA using protease digestion and labeled with an indirect immunoperoxidase staining method. IgA is clearly demonstrated.

Immunoperoxidse staining method for formalin-fixed paraffin wax-embedded renal biopsies

Most cases of renal disease affect the glomeruli. This is an important technique, particularly if glomeruli are absent in the portion selected for fluorescence and electron microscopy, but are found in the paraffin wax-processed sample. It is essential that proteolytic digestion is employed. An indirect peroxidase labeling system is recommended as this avoids endogenous biotin staining. Relatively inexpensive polyclonal antibodies, used at extremely high dilutions, can be used effectively in this technique (Figs. 19.27 and 19.28).

The key points to the protocol are:

1. Fix for 3–24 hours in a formalin fixative e.g. 10% formal saline or 10% neutral buffered formalin and routinely process to paraffin wax.
2. Cut sections at 3–4 μm onto Superfrost Plus slides and dry overnight at 37°C.
3. Proteolytic digestion is necessary and is best achieved by using 0.1% protease type XXIV (Sigma) for 45 minutes.
4. Non-immune serum is essential, especially when polyclonal antibodies are employed.
5. Polyclonal antibodies, as described in Table 19.5, are used at high dilutions for 60 minutes followed by swine anti-rabbit peroxidase-labeled secondary antibody for 25 minutes. A high quality DAB is employed as the chromogen.

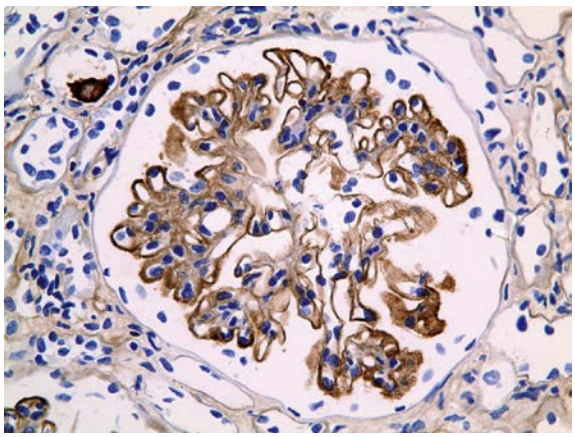

Fig. 19.28 Demonstration of IgG membranous nephropathy in a formalin-fixed paraffin-embedded renal biopsy.

Immunoperoxidase staining method for formalin-fixed paraffin wax-embedded skin biopsies

This technique may be effective on paraffin sections, however, it must be considered one of the most awkward methods employed by immunohistochemists today. Direct immunofluorescence on frozen sections is generally preferred as its relatively low level of sensitivity, compared with avidin-biotin peroxidase, reduces the labeling of non-specifically bound immunoglobulins and complement in the various tissue elements. The following immunoperoxidase protocol has proved to be successful in a number of cases, but is not always reliable.

Table 19.5 Immunoperoxidase staining in renal biopsies

Antibody	Species	Supplier	Dilution	Pretreatment
IgA	Rabbit	Dako	1/20,000	Protease
IgM	Rabbit	Dako	1/500	Protease
IgG	Rabbit	Dako	1/20,000	Protease
C3c	Rabbit	Dako	1/800	Protease
Clq	Rabbit	Dako	1/400	Protease
Fibrinogen	Rabbit	Dako	1/30,000	Protease
Kappa	Rabbit	Dako	1/20,000	Protease

Table 19.6 Immunoperoxidase staining in skin biopsies

Antibody	Species	Supplier	Dilution	Pretreatment
IgA	Rabbit	Dako	1/25,000	Protease
IgG	Rabbit	Dako	1/25,000	Protease
IgM	Rabbit	Dako	1/20,000	Protease
C1q	Rabbit	Dako	1/10,000	Protease
C3c	Rabbit	Dako	1/20,000	Protease
Fibrinogen	Rabbit	Dako	1/30,000	Protease
Lambda	Rabbit	Dako	1/20,000	Protease

The key points to the protocol are:

1. Fix for 3–24 hours in formalin, e.g. 10% formal saline or 10% neutral buffered formalin, and routinely process to paraffin wax.
2. Cut sections at 3–4 μm onto Superfrost Plus slides and dry overnight at 37°C.
3. Treat sections with 0.1% protease XXIV (Sigma) in Tris-buffered saline, pH 7.6, for 10 minutes.
4. Minimize non-specific binding by treating sections with 10% casein solution (Vector Laboratories) for 10 minutes.
5. Incubate sections in primary antibody at the dilutions shown in Table 19.6 for 30 minutes.
6. Treat sections with Dako EnVision reagent for 30 minutes.
7. Visualize with DAB for 5 minutes.
8. Positive controls for each particular antibody should be used and it is essential that normal skin should always be employed to monitor the background levels.

Note

This highly sensitive method ensures good labeling of dilute primary antibody binding to the target. The background levels of normal non-specifically bound immunoglobulins and complement are subdued. This latter point is achieved because the high dilutions of primary antibody, coupled with the short incubation times and Tween 20 TBS washes, ensure weak labeling of the less-concentrated non-specific uptake staining which so often causes considerable interpretation difficulties. In Fig. 19.29 the basement membrane is quite clearly positive for IgG.

Immunofluorescence staining procedure for frozen sections of skin and renal biopsies

Biopsy tissue should be transported to the laboratory in Michel transport medium at pH 7.0 (Michel et al., 1972) and processed for freezing as soon as possible by washing in biopsy wash solution. The tissue

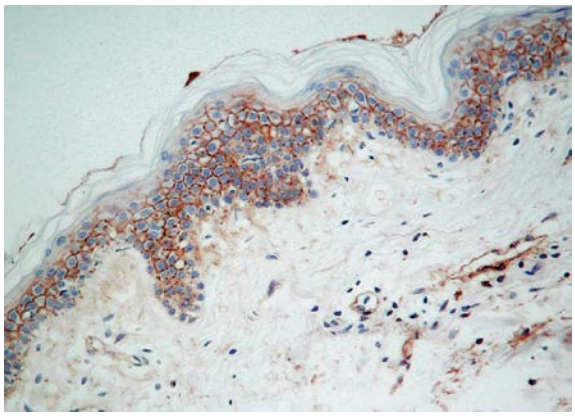

Fig. 19.29 Formalin-fixed paraffin wax-processed section of skin with a bullous pemphigoid blister, immunostained for IgG using protease digestion and an indirect technique. The bullous lesion and basement membrane clearly show IgG deposition.

can remain in Michel medium for several days, but extended time in the transport medium can increase autofluorescence and background staining (Carson, 1997). The Michel medium should be kept in capped containers and the pH checked at regular intervals as it can absorb CO_2 and become acidic, resulting in variable staining and causing other tissue artifacts.

Method for preparing frozen tissue sections

1. The biopsy should be carefully removed from the Michel transport medium with forceps and placed in biopsy wash solution for a minimum of 30 minutes. The Michel medium reversibly denatures proteins within the tissue and the washing step restores the proteins to their former state.
2. Remove the tissue from the wash solution and place onto a glass slide to remove excess wash solution.
3. A small amount of OCT compound is placed on a metal chuck which has been cooled to –25°C. The biopsy tissue is then placed in the OCT and oriented so that it is on a level plane. The OCT turns from transparent to a dense white as it freezes. Before the OCT freezes on its upper surface more is added to form a mound over the top of the tissue. This ensures that the biopsy is completely covered and has some protection from dehydration.
4. The chuck with frozen OCT and biopsy tissue should be left at –25°C for at least 30 minutes before cutting. The chuck can be wrapped in aluminum foil and stored at –20°C if sectioning is to be delayed.
5. The covering OCT is removed by sectioning at 15 µm until the tissue is exposed.
6. The tissue should be cut in 5 µm sections and attached to clean microscope slides that are at ambient temperature. One or two sections are attached to each slide, depending on laboratory protocols.
7. For renal biopsies the sections are checked at regular intervals for the presence of glomeruli by staining with toluidine blue and viewed under a light microscope. Skin biopsy sections should be checked for the presence of an epidermal layer.
8. Sections should be allowed to air dry for a minimum of 30 minutes.

Notes

a. Frozen tissue sections should be cut from unfixed tissue which has been snap frozen. Slow freezing can cause ice crystal formation, which can distort tissue morphology and antigen structure and so should be avoided.
b. The use of positively charged slides may help in the adherence of the section to the slide and prevent the section floating free or becoming fragmented during the washing stages.
c. The slides are wrapped in aluminum foil and stored at –20°C until ready for staining.

Biopsy staining procedure for both direct and indirect immunofluorescence

1. Circle the biopsy section on the slide with an isolator hydrophobic marker pen to prevent mixing of adjacent antiserum and label the slide with the antibody specificity.
2. Wash the slide in phosphate buffered saline (PBS) for 5 minutes.
3. Remove slides from the wash tank and remove residual PBS by tapping the edge of the slide against a pad of tissue paper. The slide should be wiped around the isolator ring if required but care should be taken not to wipe the section off the slide. The section should not be allowed to dry out.
4. Flood the section with working-strength antibody or conjugated antibody and incubate for 30 minutes.
5. Wash the slides in PBS for 10 minutes.
6. Remove slides from the wash tank and remove residual PBS by tapping the edge of the slide against a pad of tissue paper. The slide should be wiped around the isolator ring if required,

but care should be taken not to wipe the section off the slide. The section should not be allowed to dry out. If the sections have been stained with conjugated antiserum (i.e. direct immunofluorescence) then proceed to step 10 or step 7 if incubated with unconjugated antiserum (i.e. indirect immunofluorescence).

7. Wash the slides in PBS for 10 minutes.
8. Flood the section with the second stage conjugated antibody and incubate for 30 minutes.
9. Wash the slides in PBS for 10 minutes.
10. Mount all slides in buffered glycerol using coverslips.
11. Store the slides at 4°C until review.

Immunofluorescence microscopy

The fluorescent microscope should deliver light of a specific wavelength to cause excitation of the fluorochrome and then collect the emitted light for viewing through the eyepiece. This is achieved by applying excitation energy at the maximum absorption wavelength of the fluorochrome so that the maximum amount of light is emitted. The light source and filter arrangement of the microscope are important factors in achieving satisfactory results.

The energy output of a fluorochrome is relatively low and a light source capable of delivering sufficient excitation wavelength photons to produce visible fluorescence is essential. Many fluorescent microscopes use mercury vapor or xenon as a light source and these are contained in quartz capsules under pressure. The light output for both of these bulbs diminishes over time due to blackening of the quartz capsule and a change in the spectral emission profile. High pressure capsules are explosive and this risk increases the longer the bulb is in use; to avoid this and the added danger of mercury contamination, the capsule should be changed every 200–300 hours. If a mercury light source is used, it should be regularly checked for uniform fluorescence across the microscope field and the bulb realigned if this is not satisfactory.

Recently, light-emitting diodes (LEDs) have been introduced as a light source for immunofluorescence microscopy. These have several advantages over mercury bulbs: they do not produce large quantities of heat, have a lifetime of 8000–10,000 hours, present no explosion risk, are non-toxic and do not have warm-up or cool-down times. LED light sources are capable of producing high intensity monochromatic light and do not require bulb realignment.

Excitation and emission filters act complementary to each other and have transmission ranges which are appropriate for the fluorochrome being used. Colored glass filters were originally used but these have been superseded by broad or narrow-band interference filters. Unlike glass filters, the interference filters have a high transmission and a near vertical cut-off. The excitation filter allows light corresponding to the excitation wavelength of the fluorochrome to be directed onto the tissue section and all other wavelengths removed. Microscopes with a monochromatic LED source do not require excitation filters. Barrier filters are selected to absorb reflected excitation light and prevent it reaching the eyepieces but allow transmission of the emitted light from the fluorochrome.

Most fluorescent microscopes use a system of epi-illumination rather than transmitted light dark-ground illumination. The excitation light in epi-illumination systems is directed through the objective lens and onto the section. Fluorescent light passes from the sample through the same objective lens and is viewed through the eyepiece. A dichroic mirror between the objective and eyepiece allows selective reflection of the excitation light onto the section and then selective transmission of the fluorescent light wavelength only. The dichroic mirror only allows excitation light to pass one way and so prevents reflection of this wavelength from the tissue sample back to the eyepiece. The objective lens also acts as a condenser, so the area viewed is equal to the area illuminated and so increasing the objective magnification will give a brighter image and more fluorescence intensity. Epi-illumination has several advantages over transmitted light illumination: the light path passes through fewer glass surfaces and so less light is lost, oil immersion of the objective lens is not required, and the use of excitation and barrier filters with a dichroic mirror allows rapid changeover if a two-color fluorescence technique is being used.

A drawback of immunofluorescence microscopy is that fluorescence from the chromophore fades with time, especially if it is exposed to excitation light

for extended periods. The slides cannot therefore be used to provide a permanent record of the staining results and so photographic documentation/digitization of the tissue sections is necessary. Good images can be obtained where the sections show unequivocal staining against a darker background. Anti-fade reagents in the mounting medium reduce the rate of fluorescence fade and so can give shorter exposure time, which is especially useful if multiple exposures of a particular area of tissue are required. The camera system attached to the fluorescent microscope should ideally allow all the available light to enter the camera and not employ beam splitters for simultaneous observation and photography with the subsequent reduction in available light.

Immunohistochemical staining techniques

Avidin-biotin techniques

In these techniques either peroxidase or alkaline phosphatase may be used as the enzyme label.

Labeled streptavidin/streptavidin-biotin complex technique for monoclonal antibodies

Method

1. Rinse sections in TBS, then incubate in 10% casein solution for 10 minutes.
2. Drain off excess casein.
3. Incubate in optimally diluted primary antibody for 60 minutes.
4. Wash slides in TBS.
5. Incubate in optimally diluted biotinylated secondary antibody for 30 minutes.
6. Wash slides in TBS.
7. Incubate in optimally prepared labeled streptavidin or streptavidin-biotin complex for 30 minutes. When using a streptavidin-biotin complex the reagents should be mixed 30 minutes before use in order for the complex to form.
8. Wash slides in TBS.
9. Incubate in DAB substrate solution.
10. Wash in running water, counterstain in hematoxylin, dehydrate, clear and mount.

Note

a. Modifications required for rabbit primary antibody: in step 5 change to biotinylated swine anti-rabbit secondary. Many of the commercial streptavidin-biotin kits are supplied with a multi-species secondary link which can be used for both mouse and rabbit primary antibodies.

b. When using a goat primary antibody an appropriate biotinylated secondary antibody raised against the goat species must be used.

Polymer techniques

Dako EnVision detection technique

Method

1. Rinse sections in TBS, then incubate in 10% casein solution for 10 minutes.
2. Drain off excess casein.
3. Apply optimally diluted primary monoclonal antibody for 60 minutes.
4. Wash slides in TBS.
5. Incubate with EnVision polymer reagent for 30 minutes.
6. Wash slides in TBS.
7. Incubate in freshly prepared DAB solution for 10 minutes.
8. Rinse in TBS and transfer to running water.
9. Counterstain in hematoxylin, dehydrate, clear and mount.

Novolink polymer detection technique

Method

1. Following antigen retrieval, rinse sections in TBS.
2. Drain off excess TBS and block endogenous peroxidase activity using peroxidase block for 5 minutes.
3. Wash in TBS for 5 minutes.
4. Incubate with protein block for 5 minutes.
5. Wash in TBS.
6. Apply optimally diluted primary antibody for 60 minutes.
7. Wash slides in TBS.
8. Incubate with post primary block for 30 minutes.
9. Wash slides in TBS.
10. Incubate with Novolink polymer for 30 minutes.
11. Wash slides in TBS.
12. Incubate in freshly prepared DAB solution for 10 minutes.
13. Rinse in TBS and transfer to running water.
14. Counterstain in hematoxylin, dehydrate, clear and mount.

Alkaline phosphatase technique

Alkaline phosphatase-anti-alkaline phosphatase (APAAP) for monoclonal antibodies

Method

1. Rinse sections in TBS.
2. Drain off excess TBS and incubate in 10% casein for 10 minutes.
3. Incubate in primary antibody at optimal dilution for 30–40 minutes.
4. Wash in TBS.
5. Incubate in optimally diluted unconjugated rabbit anti-mouse bridge antibody for 30 minutes.
6. Wash in TBS.
7. Incubate in APAAP complex at the optimal dilution for 30 minutes.
8. Wash in TBS.
9. Incubate in substrate medium of choice, e.g. fast red solution.
10. Wash in running tap water.
11. Counterstain and mount as desired.

Notes

a. The reaction end product may be enhanced by repeating steps 5–8 once or twice with a reduction of the incubation times to 10 minutes.

b. As the alkaline phosphatase label is usually intestinal, it is resistant to blocking with levamisole at the concentrations described and hence it is included in the substrate mixture. Levamisole blocks most other types of alkaline phosphatase.

More detailed descriptions of these and various other methods are available in many books published on IHC methodology (see Further reading). Data sheets and instructions with the wide range of commercially available substrates/chromogens are included with the products.

Quality control in immunohistochemistry

Introduction

Since its introduction into routine histopathology in the 1980s, IHC has become established as an integral part of the diagnostic process. The use of IHC staining has developed within the diagnostic arena of an increasing range of infectious, neoplastic and reactive disease processes.

The validation of both the reagents and the results has taken on greater significance with the move towards UK accreditation with the United Kingdom accreditation service (UKAS) and other similar international accreditation bodies. The way we use and look at controls is changing. The move to automation with continual processing means it is now less appropriate to use batch controls. There is now also a focus towards finding ways of standardizing the controls themselves, and making them more clinically relevant (Torlakovic et al., 2015). This is particularly important in the control of biomarker tests. For those tests where the level of expression is variable, it is important to use controls which contain at least a high and low expressing example of the target protein. Examples of this include estrogen and EGFR receptor status where it is now mandatory to provide an in-house control with multiple expression levels for laboratories participating in the United Kingdom National External Quality Assurance Scheme (UKNEQAS). It is essential that any method using IHC principles include controls to test for the specificity of the antibodies involved, and this should be performed as part of the initial work up and validation of the antibody being tested. Polyclonal antibodies usually contain antibodies specific for several antigenic determinants on the antigen and, as many related molecules have components in common e.g. gastrin and cholecystokinin, false positive results may be obtained. Monoclonal antibodies potentially eliminate this problem but epitope similarities are seen between some molecules and unwanted cross-reaction can occur.

The criteria for specificity have been outlined by Nairn (1976), and problems relating to specificity discussed by Petrusz et al. (1976, 1977). In general, for IHC staining to be specific it must be shown firstly that no staining occurs in the absence of the primary antiserum; secondly that staining is inhibited by adsorption of the primary antibody with the relevant antigen prior to its use, but not by adsorption with other related or unrelated antigens. In practice, to evaluate the results of IHC staining, several immunological and non-immunological specificity controls may be undertaken.

Immunohistochemistry is now also able to provide prognostic or predictive information such as the likely response to specific treatments. Examples of such 'pharmaco-diagnostic' markers include estrogen (ER) and progesterone (PR) receptors and HER2/neu overexpression as well as CD117 (c-Kit) and CD20.

Reflecting the increasing diagnostic and prognostic role played by IHC, the markers are known to generate results which may directly impact upon the patient care pathway. Consequently, it is vital that these investigative procedures are properly monitored for both internal and external quality control purposes, the latter if the department is performing these tests for other laboratories.

External quality monitoring of clinical laboratories is achieved by participation in an external quality assurance scheme, such as UKNEQAS and NordiQC. Whilst in some countries participation in such schemes is a mandatory requirement in order to gain laboratory accreditation, the function of these schemes is primarily to monitor and improve the performance of the participating laboratory over a range of IHC tests.

It is important to ensure the correct internal quality control measures are in place. IHC is a complicated process and it is essential that the laboratory scientist has a sound understanding of all the requirements and procedures involved. There should be staff experienced in identifying and resolving associated diagnostic procedural problems in order to provide effective and efficient quality control within the laboratory. In addition to technical understanding, the laboratory scientist should also have knowledge of the expected staining patterns for the antibodies in both pathological and non-pathological tissues. Good communication between the laboratory scientist and the pathologist must be maintained, particularly during the introduction and validation of new antibodies and procurement of positive control material.

Detailed documentation and an audit trail throughout the process are necessary for potential back-tracking and troubleshooting. Such audit trail details can include antigen retrieval methods, antibody dilution data, control tissue samples, temperatures and incubation times. The advent of automated platforms for IHC has improved this aspect of quality control, but vigilance is still required. These automated platforms generally use standardized protocols for antigen retrieval and staining procedures, which makes overall control of the process easier. The generation and storage of automated run logs by these platforms make full reagent traceability possible. The logs can also be interrogated in the event of abnormal staining to identify errors such as missed steps due to low reagent levels.

Factors affecting stain quality

Tissue factors

Fixation

Tissue fixation has a significant influence on IHC as most antigens are altered during this process (Williams et al., 1997). The purpose of fixation is to preserve tissue and prevent further degradation by the action of tissue enzymes or microorganisms. As discussed in Chapter 4, good fixation requires tissue to have adequate time in the fixative to allow the solution to penetrate whilst retaining uniform cellular detail throughout the tissue. However, in the routine laboratory this ideal may be compromised as it is difficult to define a standard tissue size, fixation time and fixative for each specimen type. Tissues need to be adequately, but not over-fixed, so that antigenicity is preserved without excessive alteration of the protein structures of the tissue. Prolonged fixation can result in the irretrievable loss of many antigens, particularly membrane-associated antigens such as CD20 and immunoglobulin (Ig) light chains (Miller et al., 1995a; Ashton-Key et al., 1996). Lack of adequate fixation, or delay in fixation, may also be equally detrimental to labile antigens (Donhuijsen et al., 1990; von Wasielewski et al., 1998; GEFPICS-FNCLCC, 1999).

The fixatives must be compatible with IHC staining methods and formalin is still the most universally used. Formulations differ between laboratories and include 10% neutral buffered formalin (NBF), 10% formalin in tap water, 10% formal saline, and 10% NBF with saline (Angel

et al., 1989; Williams, 1993; Williams et al., 1997). Even though it may be the pathologist's choice, it can create a challenge for demonstrating certain antigens. Dabbs (2006) characterizes formalin: '*as a satisfactory fixative for both morphology and immunohistochemistry provided that a simple and effective antigen retrieval technique is available to recover those antigens that are diminished or modified.*'

Williams et al. (1997) investigated the effect of fixation on immunostaining to establish whether a specific preparation schedule would allow for the optimal demonstration of all antigens. Of the fixatives tested, 10% formal saline, 10% NBF (except for CD45RO), and 10% zinc formalin (except for CD3) gave the most consistent results overall and showed excellent antigen preservation. Recently, alcohol-based fixatives have been considered as an alternative to formalin (van Essen et al., 2010) and produced satisfactory IHC staining. Other fixatives which may still be used in some laboratories include Bouin's, B5 (mercury), zinc formalin, 10% formal-acetic and Carson's, which also have an influence on the reproducibility of staining, each presenting a change in pH, length of required exposure and different artifacts.

Fixatives dictate many factors for IHC staining, such as dilution rate, antibody incubation time, retrieval method (if applicable), type of retrieval solution and special pretreatments e.g. pigment removal. Depending upon the type of fixative used, the protocols may require slight modifications. With the advent of heat-induced epitope retrieval (HIER) (Shi et al., 1991) many of the problems associated with fixation have been reduced and, in conjunction with automated techniques, good quality staining is achievable on most tissue sections.

Processing

All tissue must also be appropriately processed to produce successful IHC staining. Tissue which is inadequately processed will potentially produce poor quality sections, with poor adhesion to the slides, especially fatty tissue such as breast and skin. Modern tissue processors all have the option to include vacuum and temperature variation at each step, allowing greater optimization of the procedure. However, high temperatures can be detrimental to antigens which are heat labile. It is recommended that paraffin wax with a low temperature melting point be used for this reason.

There is no standard paraffin wax processing protocol for the optimal demonstration of all antigens (Williams et al., 1997). In a study of laboratories in the UK, Williams (1993) found nearly as many different schedules as the number of laboratories participating in the survey. Of the nine tissue processing factors investigated, only two had any significant effect on immunoreactivity; increasing the temperature of processing from ambient to 45°C and longer processing times for dehydration and wax infiltration. Other factors including the processor, type and quality of reagents, time in clearing agent and use of vacuum, had been suggested as possible causes of poor processing (Horikawa et al., 1976; Trevisan et al., 1982; Anderson, 1988; Slater, 1988), but were found to have no effect on subsequent IHC.

Microwave processing is now being introduced into some laboratories to reduce the processing time and improve turnaround times for diagnostic specimens; it has been used successfully in conjunction with routine antibody staining. Acceptable staining was achieved when compared to tissues processed in a conventional processor (Emerson et al., 2006). As with all processing, if the tissue is not completely fixed then artifacts will be introduced.

It is important that any control tissues used in the laboratory are processed using the same protocols used for the patient samples.

Reversal of fixation/epitope retrieval

The quality and reproducibility of IHC staining relies upon the reversal of the cross-linking of proteins by the fixation process. This results in the targeted epitope being exposed, allowing the antigen binding site to be available to the primary antibody. The revolution of reversing the hydrogen cross-bonds formed by formalin was introduced by Shi et al. (1991). There are now numerous methods for epitope retrieval including enzyme digestion techniques or, more commonly, heating the slides in a buffered solution. Cattoretti et al. (1993) introduced

the solution most commonly used in standardized retrieval methods. They used a citrate buffer at pH 6.0, which is inexpensive, stores easily, and is readily available commercially or easily prepared in the laboratory. Other buffers used include EDTA-based solutions at a higher pH range, these produce more intense staining with some antibodies. These methods all successfully demonstrate a much greater range of antigens in tumors, including proliferation markers and oncogene expression. The use of automated immunostainers has brought greater standardization of retrieval methods, as these use standard retrieval solutions with defined reproducible protocols. Non-automated laboratories may have a number of variables which require internal standardization in the antigen retrieval technique, including the choice of heating method (e.g. pressure cooker, microwave), retrieval solution, pH, temperature, volume of the fluid, and the temperature and exposure time whilst heating and cooling slides.

Equipment commonly used to perform epitope retrieval includes the modified pressure cooker, initially reported by Norton et al. (1994), microwaves, water bath or a pretreatment module. Some automated platforms have on-board retrieval where individual slide bays can be heated with the appropriate solution on the slide.

Other factors required for successful retrieval include the proper drying and complete removal of water from slides. In addition to avoiding wrinkles or tears in the tissue, these factors will all assist the adhesion of the tissue to the slide. In enzymatic proteolytic 'epitope retrieval' e.g. trypsin digestion, the choice of enzyme usually dictates the temperature and pH of the solution, as different enzymes have different preferential pH and temperatures. For example, the optimal values for a mammalian-derived trypsin are pH 7.8 at 37°C, with 0.1% calcium chloride included as an activator (Huang et al., 1976). The concentration of enzyme required is dependent on the proteolytic qualities of the product being used. A typical concentration of 0.1% is used for many commercial trypsins employed in IHC. The concentration, pH, and temperature are then usually held constant, while the time of digestion is varied. The time required for optimal digestion will vary, depending on the antigen under investigation, the quality (proteolytic capabilities) of the trypsin and the length of formalin fixation. The time for optimal digestion of antigens which are only present in small amounts, e.g. immunoglobulin light chains on the surface of B cells, may vary from case to case, depending on how long each case has been fixed in formalin.

Reagent factors

Production of high quality staining is dependent upon the correct storage, handling and application of the reagents used. Once a protocol has been developed, it is important to ensure the reproducibility of the stain. To achieve this, the storage conditions and expiration dates of in-house and commercial reagents must be monitored as the preparation and use of each reagent must be consistent. Details of the storage and preparation of all reagents used in each staining run must be documented as part of the audit trail to allow backtracking and troubleshooting.

Reagent monitoring is one area in which the use of an automated staining system with barcode reagent labeling can be of assistance. These systems alert the operator to reagents which have reached their expiry date and create an audit trail with quality control documentation.

Buffers and diluents

The buffer and diluent used for wash steps and antibody dilution will affect the results of immunostaining. The pH of these reagents needs to be monitored and must be checked and documented prior to use. If it falls outside of the range prescribed by the established protocol, corrections must be made or the reagent discarded.

Many antibody diluents contain additives such as sodium azide to stabilize and maintain the protein. Although this extends the shelf life of the antibody, the additives may interfere with, or inhibit, staining if present at excessive levels.

Antibodies

The storage temperature of an antibody is critical to its stability. Commercially available antibodies

should be accompanied by a specification sheet, containing storage and handling instructions. Concentrated antibodies tend to have a longer shelf life than pre-diluted 'ready to use' antibody preparations. Concentrated antibodies can be mixed with glycerine to prevent ice crystal formation, aliquoted into cryovials, then 'snap' frozen and stored in a −80°C freezer. This greatly extends their shelf life but compliance with the expiry date printed on the antibody packaging is important. The storage temperature for antibodies and reagents should also be monitored closely, as any fluctuation in temperature may cause increased deterioration of reagents. Frost free −20°C freezers should be avoided for the storage of antibodies due to the damage caused by the freeze-thaw cycles that these types of freezer perform.

Procedural factors

The automation of immunostaining is perhaps the easiest way of improving the reproducibility and consistency of the staining. Although the use of automation is increasing, many laboratories still perform at least some IHC staining manually. Other laboratories perform semi-automated staining, e.g. manual epitope retrieval. The production of good manual procedures is important for these stains and also for use in case of failure of automated staining machinery. These procedures must be clear, easy to follow and sufficiently detailed to ensure a minimum of inter-operative variation. Adherence to these protocols and the reduction of human error is the goal in order to ensure consistent stain quality.

Block and slide storage conditions

Processed blocks should be kept in a cool dry place. Resealing paraffin wax blocks following cutting can help protect the tissue from everyday elements such as air drying, excessive moisture or physical damage. The use of fresh cut controls for each run would be ideal but this is not always feasible in a busy laboratory. Repeated sectioning of a control tissue block can also result in loss of usable tissue, although some laboratories now have a control on the same slide as the test section.

Pre-cutting control slides is more time efficient and serial sections result in minimal tissue loss. The correct storage of pre-cut slides is important and frequently overlooked as a potential source of error in staining. Some studies have found deterioration of antigens in stored sections (Raymond & Leong, 1990; Bromley et al., 1994; Prioleau & Schnitt, 1995). Others have found no deterioration of some markers investigated, including ER, CD3, CD20, CD45RO, vimentin and immunoglobulin light chains in sections stored for up to four months at room temperature (Williams et al., 1997; Eisen & Goldstein, 1999).

The viability of the antigen and speed of antigen deterioration in cut tissue sections is highly dependent upon the antigen under consideration and the temperature used for section adhesion. For example, whilst antigens such as CD30 and PSA seem relatively robust, CD117 (C-kit) deteriorates quickly and should be cut fresh.

The temperature at which the slides are dried can also affect the immunoreactivity of the antigens. It is advisable to dry all slides for IHC staining at 37°C. Urgent cases can generally be dried at 60°C for up to 4 hours. The exception to this is HER2, where it is recommended that sections should not be dried at 60°C for more than 1 hour.

An appropriate number of control slides and blocks should be kept with these factors in mind. This stock level will vary depending on the workload in each individual laboratory.

Monitoring stain quality

Whenever a new batch of reagent is used, its details and the efficacy must be recorded, checked and compared with the previous batch. Reagents from different batches should never be mixed. Any enzymes used must be validated prior to use due to a high degree of inter-lot variation. It should be noted that poor storage or shipping conditions can result in a reduction in enzyme activity. Antibody validation documentation should be available and is best kept in combination with the antibody specification sheet. This may be in either paper or electronic form. Detection system reagent validation is best performed by using a panel of several different antibodies. This should include different antigen retrieval

methods and cover a variety of different staining patterns; nuclear, cytoplasmic and membrane staining. A clean, i.e. non-reacting negative control is just as important as the intensity of positive staining patterns, and such controls must be carefully checked.

Validation of antibodies

It is important that all antibodies used in diagnostic testing are fully validated in the laboratory and detailed records are kept of the validation process. This should include demonstration of the reactivity of the antibody, validation of the procedure and quality checking of positive and negative controls used with that antibody. Before introducing a new antibody into the laboratory repertoire it is important to research details of the clone required. Most commercial antibodies have data sheets available on-line which should include a number of facts for consideration. These should indicate the host in which the antibody was raised (e.g. rabbit, mouse, or goat), location of the target antigen, concentration of the antibody, recommended application (e.g. frozen tissue, formalin-fixed paraffin-embedded tissue), recommended positive and negative control tissue sources, classification (e.g. analyte-specific reagent, research use only, or in vitro diagnostic) and reference materials for the application of the antibody. The specification sheet also typically includes suggested staining protocols.

Most antibodies used in diagnostic pathology laboratories are classified as 'research use only' or 'analyte-specific reagent'. This means that it is the responsibility of the diagnostic laboratory to validate and document the sensitivity and specificity of the antibody. This process can be simplified if an appropriate antibody classified for 'in vitro diagnostic' (IVD) use can be sourced, where the vendor has assumed responsibility for the validation and application of these antibodies.

The suggested protocol should serve as a baseline whilst working up the antibody and each laboratory should optimize the stain. If no protocol is suggested by the manufacturer, journal articles can be a good source of baseline protocols.

Selection of the most appropriate epitope retrieval method is important to ensure the maximum sensitivity of the stain. Antibodies are pH sensitive and it has been shown that staining intensity is better when the epitope retrieval step is performed at a pH specific to each antibody. It has also been shown that an antibody may not be specific or work as well if the appropriate pH is not maintained during the retrieval step. Selection of the detection complex used may also be influenced by the primary antibody. Some antibodies have been found to work better with an alkaline phosphatase detection system than with a horseradish peroxidase system and vice versa. Antibodies commonly used in pigmented tissues such as melanoma markers may benefit from a red (or other) chromogen end-point, as the endogenous pigment can disguise the more commonly used brown DAB chromogen.

Staining protocol development generally consists of trial and error, making sequential alterations in order to achieve optimal signal-to-noise ratio. In real terms this means strong, crisp target antigen staining with little or no background staining. If the staining is too strong, then further dilution of the primary antibody may improve staining specificity. If target antigen staining fades and the background remains, then the addition of a blocking step, change of epitope retrieval method or changing the detection system used (e.g. to a polymer-based system) may improve the result. Changing the antibody diluent may help to increase the antibody's reactivity, while at the same time reducing background staining. Weak staining may be improved with an increase in the antibody concentration (e.g. from 1:50 to 1:25 dilution).

Negative staining can be more difficult to resolve and may be the result of multiple factors. It is advisable to rule out human or mechanical error by repeating the staining before making further modifications. If the stain remains negative, change one variable at a time and document each reagent, step and reaction time. Negative staining may be resolved by increasing the antibody concentration, changing the epitope retrieval method, changing the solution pH, changing the antibody diluent to one of a different pH, changing the base composition or

using an amplification step as part of the detection system. It is also important to ensure that the tissue stained should exhibit positive staining and the slides are freshly cut.

The duration of formalin fixation to which the tissue has been exposed may affect the reversal of protein cross-linking. Over-fixation of tissue may require more aggressive epitope retrieval methods to achieve satisfactory results. This must be considered if the control or test tissue available for protocol development and validation has been stored in formalin for an extended period of time.

The final staining protocol must be confirmed on both positive and negative tissue controls prior to implementation. False positive staining of negative control tissue suggests that the concentration of the antibody is too high.

Once an antibody dilution and staining method has been established and validated, each step of the procedure must be clearly documented and maintained in written or electronic form. This should include:

- The antibody lot.
- The expiration date.
- The dilutions used.
- Details of the blocking steps (serum, avidin-biotin, hydrogen peroxide).
- The secondary antibody.
- The label detection method.
- The chromogen used.
- The duration of each step.

Controls

Increasingly, these now have both test and control sections combined on the same slide and can include both reagent substitution and internal or external tissue controls. A positive control for each antibody should be used with every staining run. It is important to obtain a reliable, known, positive control tissue for use with each antibody offered by the laboratory. Control tissue selection should be supported by publications and the selection process should be documented. Many commercial antibody specification sheets make recommendations regarding controls and the most cited references which are of use when selecting control tissue.

The acquisition of appropriate control tissue requires knowledge of the desired tissue type (e.g. kidney, liver) and whether the target antigen is expressed in normal or tumor tissue. It is helpful if the person validating a control has knowledge of the prospective control tissue diagnosis in the case of tumor controls to assist in the evaluation of expected staining patterns. In order to be comparable to the test section it is important that the fixation and processing of control tissue should be the same as the patient tissue being tested. Ideally, the target antigens in positive controls should be distributed across the entire sample and, if possible, should be present in a range of densities in order to monitor the sensitivity as well as specificity of the stain.

Whilst a separate positive control should be included for each antibody, a single tissue type is often suitable for use as an external control for several different antibodies. For example, a section of appendix can be used for testing antibodies to low molecular weight cytokeratins, EMA, vimentin, desmin, SMA, CEA, S-100, NSE, CD45, CD20, CD3, CD4, CD8, CD79a, bcl-2, Ki-67, etc. (Balaton, 1999). One way of creating a control block containing multiple positive and negative tissues suitable for a wide range of antibodies is to use a tissue microarray (Figs. 19.30 and 19.31). A composite block, containing representative punches from multiple tissue types in a single block, can be used for the majority of stains offered by a laboratory. With careful tissue selection the block should not have to contain an overlarge number of punches in order to achieve this goal. A drawback of this approach is that, whilst it simplifies daily quality control, it requires a large amount of available control material, particularly in larger laboratories offering a wide range of antibodies.

Internal and external positive controls

Many tissues contain native components which serve as internal positive controls for IHC staining, e.g. the crypts of normal colon stain with polyclonal CEA (Fig. 19.32). These internal controls are a useful adjunct to external tissue controls, since they demonstrate that the test tissue was appropriately fixed and processed. Although external positive tissue controls do not show this, they are

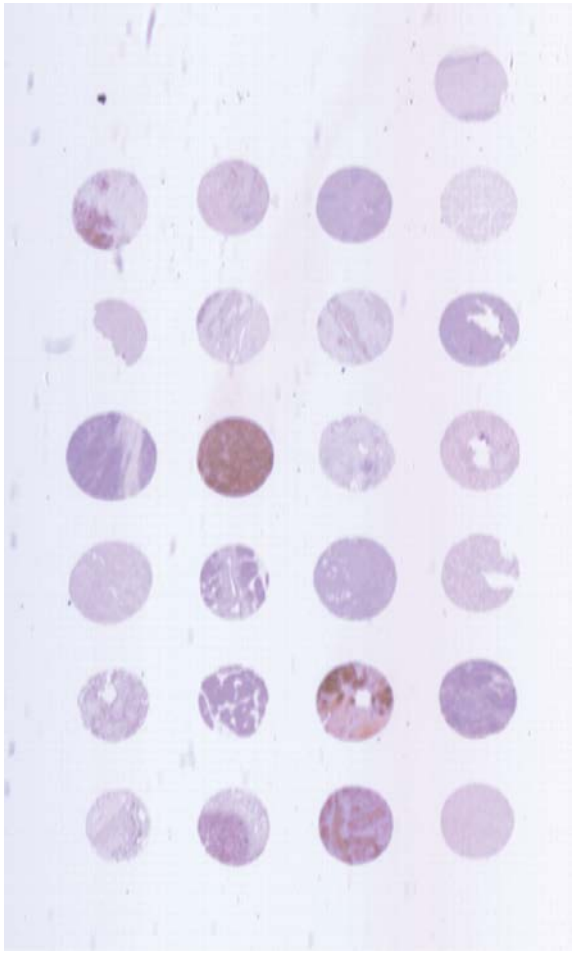

Fig. 19.30 Low-power image of a multi-tissue control stained with an antibody to polyclonal carcinoembryonic antigen (CEA). Using carefully chosen 'donor' tissues, both positive and negative tissue controls can be demonstrated on the same slide.

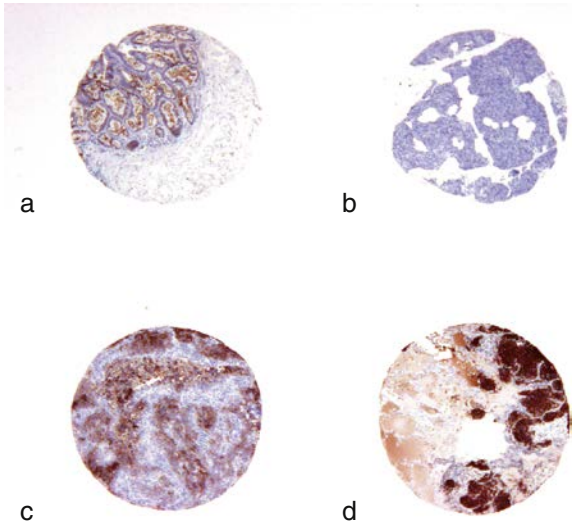

Fig. 19.31 High-power image of multi-tissue cores, control stained with an antibody to polyclonal CEA, demonstrating (**a**) normal tissue, (**b**) negative control, (**c**) and (**d**) tumor staining.

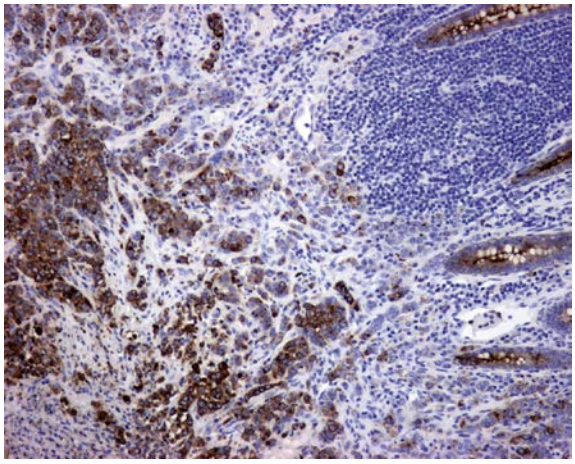

Fig. 19.32 Section of colon with carcinoma on the left, also demonstrating the staining of normal crypts on the right for polyclonal CEA. This serves as an internal positive control.

required for some tests where there is no internal positive control in the tissue under investigation, such as those performed for infectious agents. External controls are also needed whilst working up an antibody for inclusion into the laboratory repertoire. They also assist the day-to-day monitoring of the stain where it is preferable to use the same tissue with a known pattern of staining. If an internal control is not present, and/or if there is doubt over the retention of antigenicity in the test tissue, then this can be checked by staining with vimentin. However, since vimentin is a relatively robust antigen it is possible that its viability could be maintained whilst more labile antibodies such as CD3 may be lost.

Daily slide review

Ideally, all slides should be reviewed and quality assessed prior to being sent out of the laboratory. This can be performed by an appropriately trained

laboratory scientist or a pathologist. The reviewer should be able to distinguish acceptable signal-to-noise ratio and recognize interpretable results. At a minimum, the positive control slides should be screened for expected results.

Without stained controls the results of many IHC assays cannot be validated. The controls should also demonstrate the sensitivity (i.e. tissue with low expression of the target antigen stains positive) and specificity (i.e. negative controls are not stained and background staining is absent) of the stain. Controls indicate whether the staining protocols have been followed correctly, whether day-to-day and worker-to-worker variations have impacted upon stain quality and whether the reagents used continue to be in good working order.

External quality assurance

Whilst daily internal monitoring of stain quality through the use of control slides is vital, it is also important to ensure inter-laboratory consistency of quality and results. This is best achieved through participation in an external quality assurance scheme such as UKNEQAS or NordiQC. These schemes are arranged into different modules, each covering common stains performed in different areas of histopathology. Examples of modules are breast pathology (e.g. HER2, ER, PR), neuropathology (e.g. NFP, GH, TSH) and general pathology (e.g. desmin, CD3, Ki67). Participants choose which modules they wish to participate in, and are sent slides to stain with relevant selected antibodies. The stained slides are returned to the scheme organizer often accompanied by one of the laboratory's in-house control slides used for the requested antibody. Submitted slides are assessed and scored independently by the scheme organizer and participating laboratory, who will then receive both individual feedback on the slides submitted and general feedback on results from all participants. The methods used to produce the highest scoring slides are taken as 'best practice' and made available for all laboratories to consider. Any laboratory producing consistently poor results will be offered advice by the scheme organizers to help improve future results.

Through participation in such schemes the laboratory can assure service users that the results they produce are not only consistent but are also in concordance with the national consensus. This is particularly important for laboratories using prognostic and predictive markers which may impact on patient care (Rhodes et al., 2001; Ibrahim & Miller, 2008; Bartlett et al., 2009). In many countries participation in such schemes is a prerequisite for the accreditation of the laboratory.

Troubleshooting

Troubleshooting problems in IHC can be difficult due to the complexity of the technique and the number of variables involved. Good documentation of the processes involved can help with backtracking to potential sources of error. The use of barcodes on slides and reagents on automated staining systems has helped to reduce some of the common human errors, but machines are not entirely reliable and have their own associated problems. One of the advantages of an automated system is the ability to see on screen records of staining logs and reagent preparation details, these can help to identify problems promptly.

A key element to producing good quality staining is in the preparation of the slides. Poorly fixed or processed tissue tends to be more difficult to section, being prone to detachment from the slide, especially given the harsh nature of some pretreatment protocols. Furthermore, poor section quality can lead to problems with interpretation if visualization of the staining is occluded, or the tissue morphology is damaged. This includes section thickness, damage, e.g. holes or scores, and excessive heat exposure causing dry sections. An experienced laboratory scientist with a good understanding of all the processes and techniques involved should be able to identify and resolve all of the problems which may arise.

The most common problems likely to occur are either false negative or false positive staining, and the potential sources of error for these are discussed below.

False negative staining

The absence of staining of an antigen which should be present in the tissue can present in various patterns. The easiest to identify is when the positive control

slide and the patient test slide are completely negative. After checking that the correct positive control section has been used, the source of the problem needs to be identified and rectified, before the stain is repeated.

In the second type of false negative staining the positive control slide is negative, but the patient slide shows positive staining in either the area of interest, or in some internal component which acts as a positive control. If this internal control stains according to the expected pattern it may not be necessary to repeat the stain as long as the failure of the control is documented. The cause of the failure of staining in the positive control still needs to be determined to ensure it is still viable to be used in future staining runs.

The third pattern of false negative staining occurs when the positive control slide stains appropriately, but the patient slide is completely negative. This is more difficult to detect because at first glance it may appear that the patient test is just negative. In this instance it is important to check for any internal positive control components within the tissue, and if these are also negative the stain should be repeated. If the negatively stained section was part of a panel of antibody tests, then it may help with the assessment if the negative result is appropriate when compared to the other test results. A number of factors can cause false negative staining.

Process failure

This can be caused by human or mechanical error. Human errors usually occur because the standard procedure has not been followed; commonly such errors involve missing a step, performing steps out of sequence, incorrect reagent preparation, or incorrect reagent incubation. Chemical or immunological reactions may not to go to completion if steps have been shortened.

The automation of processing has cut out some potential errors by barcoding slides and reagent bottles. It is still possible for slides to be incorrectly labeled, or for reagents to be wrongly prepared unless they are commercial, ready-to-use reagents. Staining is compromised when reagents are skipped if the instrument runs out of wash buffer. Incorrect programming of an instrument can occur, but this should be picked up before a staining run is started. A common artifact is uneven staining of the section caused by incomplete coverage by one or more of the reagents (Fig. 19.33).

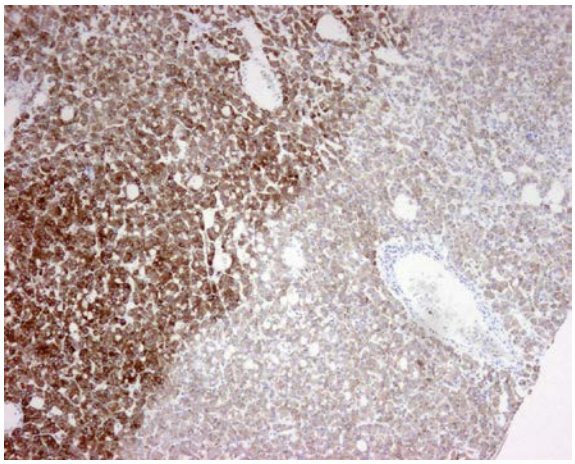

Fig. 19.33 Adenocarcinoma stained on a horizontal immunostainer with an antibody to cytokeratin 7, demonstrating incomplete coverage of the tissue section by the staining reagent.

Instrumentation failure can also occur through pump failure, clogged or damaged probes, or because electrical components wear out. The computer can have software glitches or experience external network problems if interfaced with a laboratory or hospital system. Equipment should be well maintained and regular maintenance is important to keep instruments in good working order.

Positive control selection

Positive control material should have previously been tested for validation before use. If the positive control slide is negative and the patient slide positive, there are three main problems which could have occurred:

1. The wrong control could have been used, which may happen when inexperienced staff members are working in the laboratory. It can help to clearly label precut control sections with the antibody or antibody group they can be used for, such as

cytokeratins, or to have a list of antibodies and the appropriate control section required.

2. The area of interest in the control slide has been cut through. This can be prevented by testing the first and last slide of each batch of sections, validating that the control is still demonstrating appropriate staining.
3. Some antigens are more labile, and their antigenicity deteriorates with time in cut sections, resulting in weak or negative staining. If this is suspected, a fresh section should be cut and stained to make sure the block still contains viable control tissue. Once any of these antigens have been identified or there are antibodies which are used infrequently, it is advisable for the laboratory to only keep a small stock of precut sections, or to cut them freshly as required in order to prevent further occurrence.

Incomplete deparaffinization

Failure to remove paraffin wax from the slide may interfere with the ability of the antibody to penetrate the tissue and this may inhibit antibody binding. Reagents used for de-waxing and dehydration should be changed regularly, and paraffin wax with a high plastic content may require longer times in xylene with agitation to assist with its complete removal. Some pretreatment solutions de-wax and pretreat in one step. It is important to monitor the use and temperature of the reagents and follow the manufacturer's recommendations for use to ensure that they continue to perform at an optimal level. Changing or rotating solutions regularly is recommended to ensure that deterioration of these reagents does not introduce unnecessarily weak or false negative staining, this is often overlooked as a potential problem.

Epitope retrieval

It is essential that the correct epitope retrieval protocol has been performed on the sections, as not all retrieval solutions work for all antibodies used in the laboratory. For example, the antibody BerEP4 will generally not work if pretreated in high pH EDTA-based retrieval solutions. The retrieval protocol should have been defined as part of the optimization of a new antibody and should be strictly adhered to, otherwise it is likely to result in false negative staining. Slides should be clearly labeled and sorted for the specific retrieval procedure required. This is generally less of an issue on automated platforms as the retrieval step is part of the set staining protocol. The retrieval will either be carried out on the machine, or a slide label will have been attached to the slide with the appropriate protocol details printed on it, making it easier to identify the correct pretreatment.

When HIER protocols are performed, it is advisable to monitor the temperature of the solution to check that it has reached the correct setting, and this has been maintained for the required length of time. Failure so to do may result in substandard reversal of fixation and possible negative staining. Many of the automated systems are able to monitor and log retrieval temperatures electronically and this can be easily viewed on screen.

If enzyme digestion is employed as the retrieval method, care should be taken to ensure that the correct time and temperature protocol is used depending on the digestion agent and the antibody in question. Over-digestion of the tissue can result in the loss of its morphology, at which point the antigen may have been destroyed, resulting in negative staining.

Temperature

Chemical reaction rates are affected by temperature, and it is important to monitor all aspects of the procedure where heating or cooling is required, either during pretreatment or staining. Some automated stainers use heat during various steps to speed up chemical reactions and these can develop faults. Regular maintenance and monitoring should help to prevent these occurrences. Antibodies are proteins, and as such their structure can be modified by heat which may decrease the sensitivity of antibody binding during the staining process. It is also important not to overlook the general room temperature of the laboratory where IHC staining takes place. With numerous fridges and staining machines working, the core temperature of the room can rise by several degrees if

there is no air-conditioning system in place. Some staining machine manufacturers have recommendations about minimum and maximum advised working temperatures.

Antibody preparation

All commercially available antibodies should be labeled with an expiry date and come with a datasheet detailing their correct storage requirements. Most concentrated and pre-diluted antibodies are recommended to be stored at 2–8°C. Those which are less stable may need to be aliquoted and frozen at –20°C. The efficiency of antibodies stored at 2–8°C will still decline over a period of time due to oxidation, eventually producing a false negative result. This deterioration may be picked up by close monitoring of the positive control section if the expected level of staining starts to decrease.

Human error in the preparation of an antibody dilution may occur. Pipettes should be regularly maintained and calibrated at least annually. Staff should be trained in the correct mechanical use of the pipette, as well as choosing the appropriate size of pipette for a particular volume range. Antibody dilutions should be documented to include the date that the antibody dilution is made in order to identify when a possible preparation error may have occurred.

Chromogen incompatibility

Various detection systems are available for visualization of the antibody and it is important to understand the compatibility of chromogen and enzyme label. The standard combination is horseradish peroxidase (HRP) enzyme used with diamino-benzidine (DAB) chromogen, but other chromogens such as amino ethyl carbazole (AEC) work with HRP as well. Fast red chromogen and BCIP/NBT react only with an alkaline phosphatase enzyme. Reading the manufacturer's recommendations for preparation and shelf life of a prepared chromogen is important. These problems should not occur on automated platforms, as the detection reagents are generally supplied as a ready-to-use kit with barcoded bottles which cannot be confused. When using alkaline phosphatase based detection systems, care should also be taken during the counterstaining to mounting steps as some enzyme labels are alcohol soluble and cannot be dehydrated.

False positive staining

This is often easier to troubleshoot than false negative staining, although it is potentially much more serious. If the false positive staining is interpreted by the pathologist as real, i.e. positive staining, then the patient may be incorrectly or unnecessarily treated. An experienced laboratory scientist should be able to identify possible false positive staining, and should then either repeat the test or bring it to the attention of the pathologist to discuss any action required. When false positive staining is observed in a patient slide only, it may be due to different fixation or processing of that patient tissue. In this instance, it may be helpful to run a negative patient slide alongside the repeat test to check for non-specific false positive staining. As with false negative staining, there are several factors which can cause the problem.

Poor quality of fixation

With greater emphasis on meeting targeted diagnostic turnaround times, the laboratory can be put under pressure to process samples as quickly as possible. This can greatly impact the quality of tissue processing. When fixation and processing times are too short, tissue may not be adequately dehydrated. The result is that the tissue may be partially unprocessed, or the center of the tissue will be alcohol fixed during processing. In this case the IHC staining pattern of the patient test tissue can be quite variable compared to that of the control section on which the dilution and method protocols have been developed (Fig. 19.34).

Technical preparation

As mentioned previously, it is important to start with good-quality sections for IHC staining, as this is a common cause of false positive staining. Poorly fixed and/or processed tissue is more difficult to cut and has a greater tendency to detach from the slide. All sections should be picked up onto positively charged slides to assist with tissue adhesion

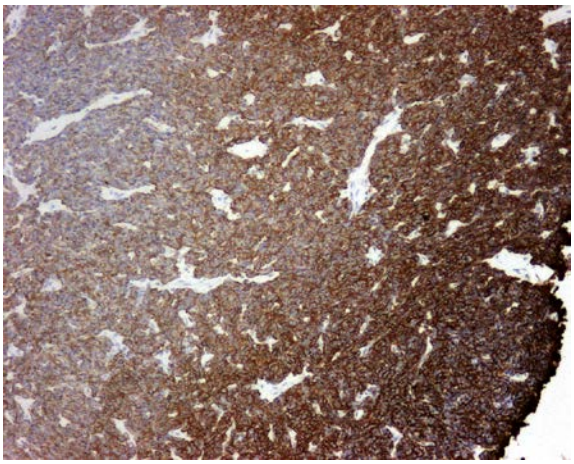

Fig. 19.34 Section of poorly fixed thymus stained with an antibody to AE1/AE3 cytokeratin cocktail, demonstrating a gradient of staining from the formalin-fixed outer edge to the alcohol-fixed center.

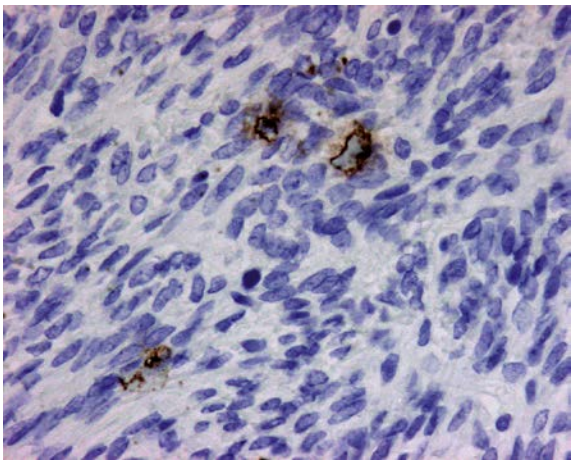

Fig. 19.35 Section of lymph node stained with an AE1/AE3 cytokeratin antibody cocktail, demonstrating squamous cell contaminants which result from the scientist placing ungloved fingers into the water bath, also known as 'floaters'.

and to prevent detachment during potentially harsh pretreatments. They should all be cut at the same thickness and need to be as flat and wrinkle free as possible. The water bath used for floating out the sections should be wiped after every case to prevent 'floaters' and squamous cells being transferred to the next section (Fig. 19.35).

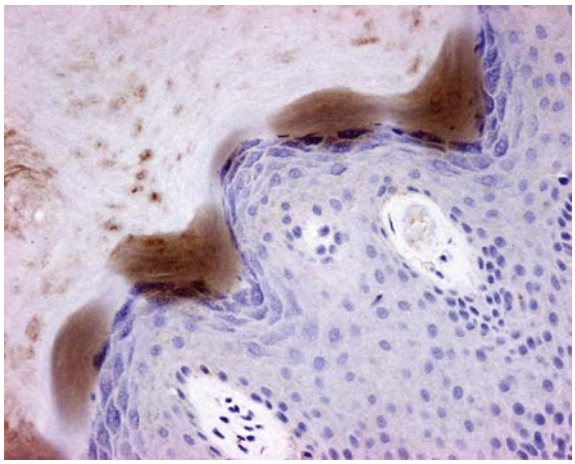

Fig. 19.36 Section of skin intended as a negative control, demonstrating trapped chromogen under the keratin layer due to tissue lifting.

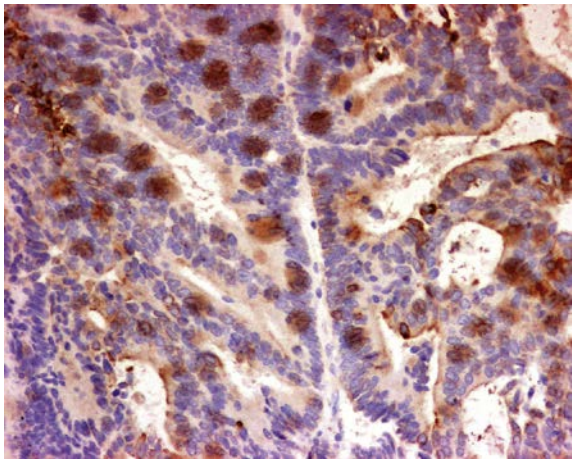

Fig. 19.37 Section of colon, demonstrating trapped chromogen due to poor tissue adhesion to the slide.

Poor section quality can result in streaks, overall blushing across the tissue, or patches of non-specific positive staining. Calcified tissue can cause scores or holes which may affect staining, but any decalcification treatment must be kept to a minimum as the acidic reagents can be deleterious to the end result. Wrinkles, tears and folds create areas in which the reagents are not properly rinsed away and remain trapped underneath or on top of the tissue (Figs. 19.36–19.38), so that by the time the chromogen is applied, those areas are

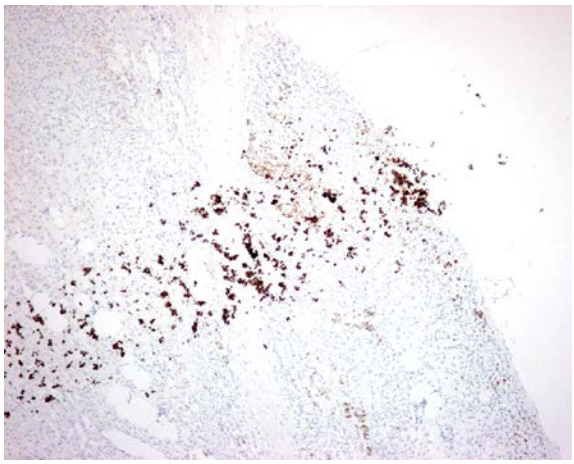

Fig. 19.38 Section of skin stained with an AE1/AE3 cytokeratin antibody cocktail, demonstrating chromogen streaking across the section due to poor rinsing. Staining is present across an area expected to be negative.

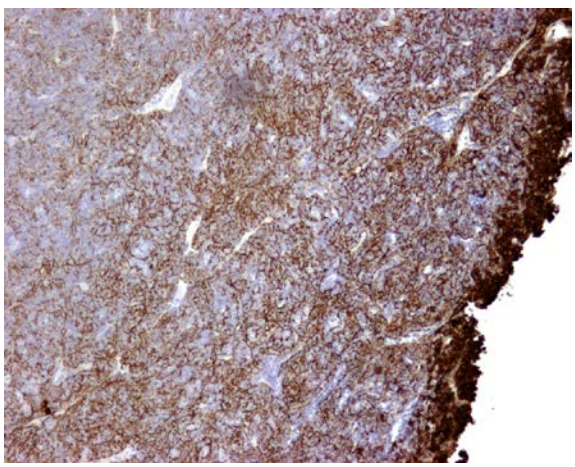

Fig. 19.39 Tissue section, demonstrating edge artifact due to excessive drying.

intensified and may make it impossible to interpret the staining.

Once cut, the sections need to be dried properly before staining to help with adhesion. Oven drying must be monitored and maintained at a constant temperature, as tissue exposed to high temperatures for long periods of time may demonstrate edge artifact (Fig. 19.39). If sections are not going to be stained immediately, they should be dried at 37°C and then stored at room temperature.

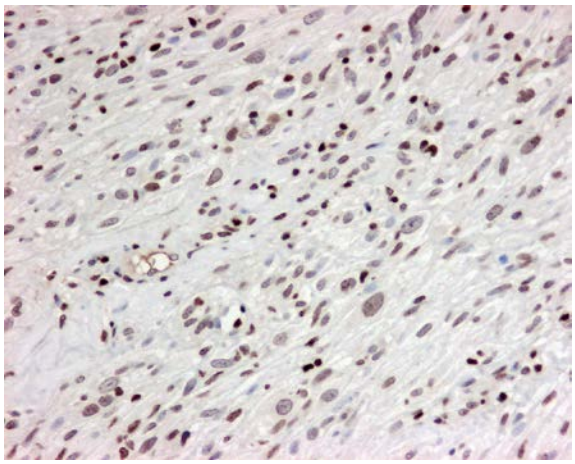

Fig. 19.40 False positive nuclear staining demonstrated with an antibody to polyclonal myosin due to unnecessary heat-induced epitope retrieval.

Epitope retrieval

The use of retrieval solutions must be monitored to ensure the correct protocol is performed each time. Variations of pH or temperature of the solution will interfere with staining results. One possible effect of incorrect retrieval is non-specific staining due to the amplification of endogenous biotin in the tissue (O'Leary, 2001), which is observed with avidin-biotin detection systems. Some antibodies do not require any pretreatment and exposure to a retrieval solution or step, may create non-specific nuclear staining (Fig. 19.40).

Care should be taken not to over-digest the tissue when using proteolytic enzymes. This can be caused by extending the time beyond that determined to be the optimal digestion time, or by performing the digestion step at a warmer temperature than has been determined in the laboratory's validation process. Excessive heat will typically increase the rate of digestion. If the section is not sufficiently rinsed following the digestion step and the enzyme is not removed completely, it will continue to digest the tissue. All of these factors lead to over-digestion of proteins, which may then diffuse into or deposit onto the tissue, leading to diffuse non-specific staining. The occurrence of over-digestion should be apparent by the subsequent damage to tissue morphology (Figs. 19.41 and 19.42).

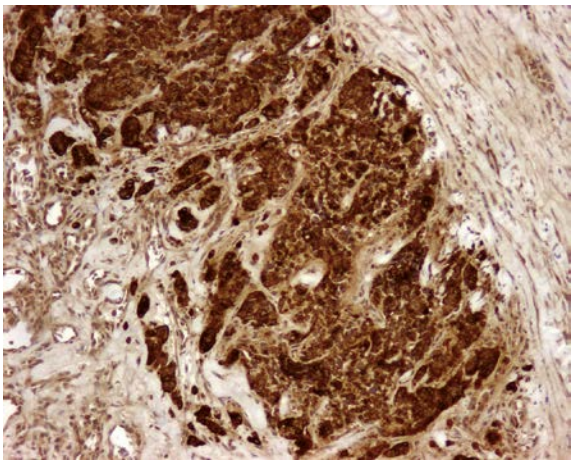

Fig. 19.41 Over-digested carcinoma stained with an AE1/AE3 cytokeratin antibody cocktail. Excessive staining creates difficulty in identifying true positive staining.

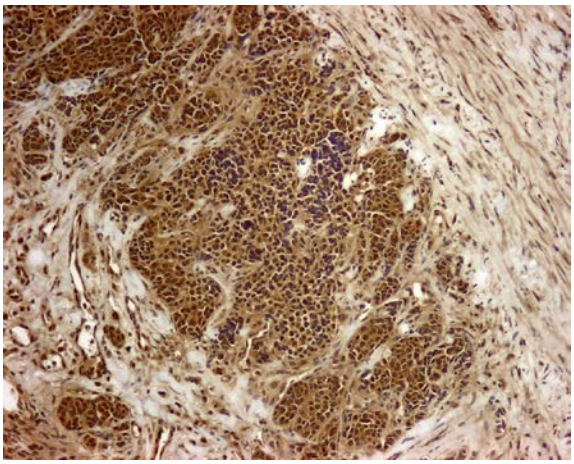

Fig. 19.42 Over-digested carcinoma (same case as in Fig. 19.41) intended as a negative reagent control slide, confirming that much of the staining observed in the patient AE1/AE3 section is non-specific staining.

Tissue drying and wetting agents

Once the tissue sections have been de-waxed they should be kept moist and fully covered by each reagent. The amount of visible artifact will depend on whether just the edges or the entire section has dried out, in the latter the whole section will demonstrate non-specific staining. Wetting agents such as detergents may be added to the rinse buffer. These detergents assist with rinsing off unbound antibodies and other reagents, keeping the staining clean. Too much of these reagents can interfere with the staining as well, so controlling the concentration in the rinsing buffer is recommended.

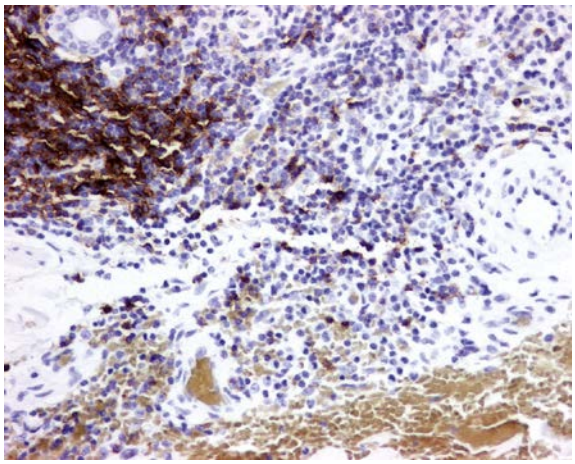

Fig. 19.43 Lymphoid tissue stained with an antibody to CD3, demonstrating non-specific staining of red blood cells (intrinsic peroxidase activity) due to inadequate quenching with hydrogen peroxide solution.

Intrinsic tissue factors

There are two main intrinsic tissue factors which need to be considered as possible causes of non-specific staining.

1. Endogenous peroxidase is commonly found in red blood cells and other tissue components (Fig. 19.43) and this can react with DAB in horseradish peroxidase detection systems if not treated. This can be blocked by treatment with a 3% hydrogen peroxide solution applied prior to staining to quench the peroxidase activity.
2. Hydrogen peroxide should be stored in dark bottles and the blocking solution should be freshly prepared before use. Staining may be observed in mast cells in a negative tissue control when inadequate quenching occurs (Fig. 19.44).

Biotin is a vitamin found in high concentration in a number of tissues, including the liver, kidney and brain. This can lead to non-specific staining when using an avidin-biotin detection system, but can be reduced by the addition of a blocking step. To achieve this, avidin is applied first for 15 minutes, rinsed with buffer, and then biotin is applied for 15 minutes, followed by another rinse in buffer. A protein block or the application of

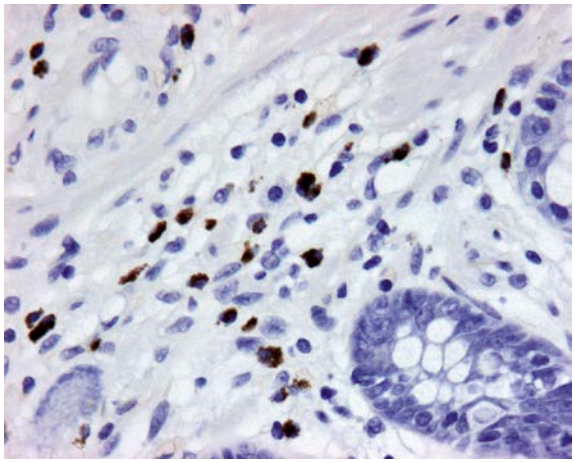

Fig. 19.44 Section of colon intended as negative reagent control, demonstrating mast cell staining due to inadequate quenching of endogenous peroxidase activity within mast cells.

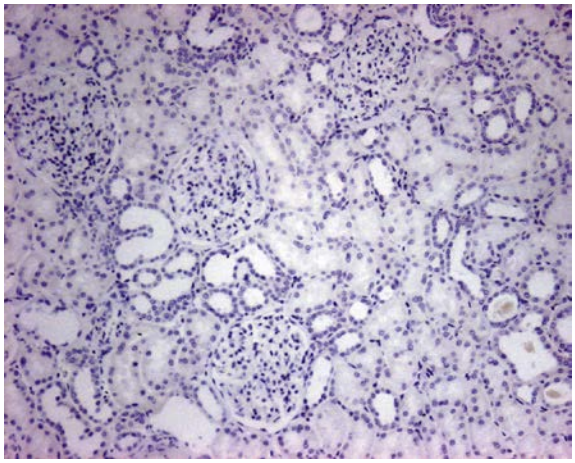

Fig. 19.46 Section of kidney (same case as in Fig. 19.45) properly blocked with avidin-biotin, demonstrating the expected absence of staining (negative reagent control).

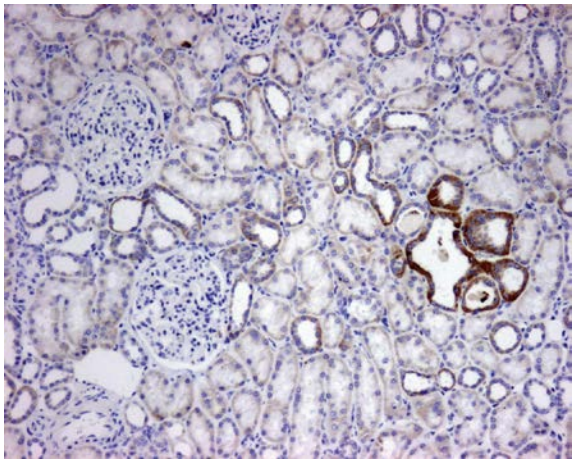

Fig. 19.45 Section of kidney intended as a negative reagent control using an avidin-biotin detection system, demonstrating non-specific biotin in some tubules.

the primary follows the avidin-biotin blocking step. Non-specific biotin will be easiest to identify in the negative control slide (Figs. 19.45 and 19.46).

Antibody concentration

All antibodies, whether concentrated or pre-diluted, should have their working dilution validated prior to implementation into the laboratory. Commercially prepared diluents are readily available and generally provide greater stability of diluted antibodies. The pH of the diluent is important to maintain the antibody in its proper structure as any deterioration can create non-specific staining and reduce the overall stain quality.

The concentration of a pre-diluted ready-to-use antibody has been determined by the manufacturer and may not have been tested with different detection systems. These antibodies should be validated before use by performing serial dilutions (e.g. 1:2, 1:4, 1:8 etc.), starting from the neat concentration, using both positive and negative control tissues. If non-specific staining is observed, the dilution should be taken out further until a good signal-to-noise ratio is achieved.

Polyclonal antibodies are more sensitive than specific. Using monoclonal antibodies reduces non-specific staining due to their specificity and affinity for one epitope.

Detection system

The choice of detection system used can impact on the quality of staining, but may be affected by other factors such as cost, or reagent rental agreements associated with the type of automated platform being used. In recent years the most commonly used system has been avidin/biotin or streptavidin/biotin, but (as mentioned above) these systems can cause problems, specifically when staining tissue types which contain intrinsic biotin. These are now being replaced by the introduction of polymer or synthetic-based systems which have allowed the elimination of biotin-induced non-specific staining.

Another advantage of the polymer-based systems is they can shorten staining times by eliminating the need for multiple blocking steps. Polymer-based systems are not a cure-all for every antibody as some polymeric complexes are large and can have difficulty reaching some epitopes, depending on their location. With the variety of detection systems now available, the laboratory has the opportunity to continually evaluate and improve the quality of the staining produced, provided it can be achieved within the financial constraints of their department.

Chromogen

Generally, chromogen solutions should be freshly prepared just before use, and if using a kit, according to the manufacturer's instructions. Commercially available products provided for use on automated stainers may be stable for extended periods of time once mixed in accordance with the manufacturers' instructions. Alkaline phosphatase chromogens are sensitive to light and heat, making them susceptible to lysis and/or loss after preparation. Peroxidase chromogens can also break down, but not as quickly. Once chromogen activity is depleted, it will either create a blush across the tissue or deposit debris. Chromogen precipitate and streaking can be reduced with adequate mixing, rinsing and filtering of the chromogen prior to application (Figs. 19.47 and 19.48).

Extended time in primary, secondary, or chromogen and inadequate rinsing with buffer between steps may also cause non-specific staining.

Species cross-reactivity

Whilst many commercial kits are available ready to use, it is important that the user should understand their formulation. Kits which use 'universal' secondary reagents are directed at many primary antibody targets. This information is especially important if staining animal tissue, e.g. goat anti-mouse IgG in the kit can cross-react with mouse tissue, creating a non-specific background stain. Mouse tissue typically will demonstrate blood vessel staining due to such cross-reactivity. This staining mimics endothelial cell positive staining. To avoid such non-specific staining, primary antibodies raised in a different species, and secondary reagents not directed against mouse IgG, should be used. If a primary antibody is to be used which originated in the same species to that being stained, special blocking steps can be introduced to minimize non-specific binding. Kits containing such blocking reagents are commercially available.

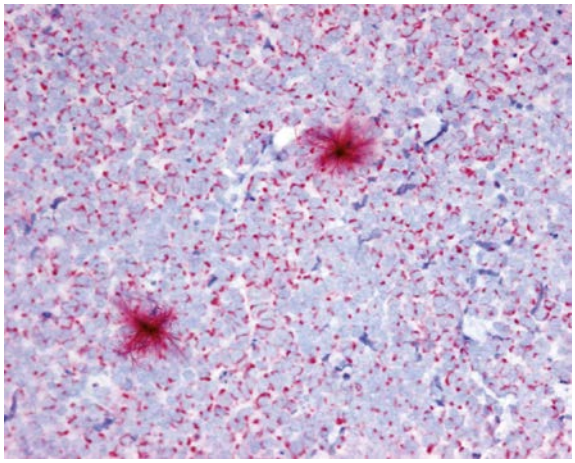

Fig. 19.47 Merkel cell tumor stained with an AE1/AE3 cytokeratin antibody cocktail using alkaline phosphatase detection with red chromogen, demonstrating crystals on the surface. Crystal deposits could have been avoided by filtering the chromogen.

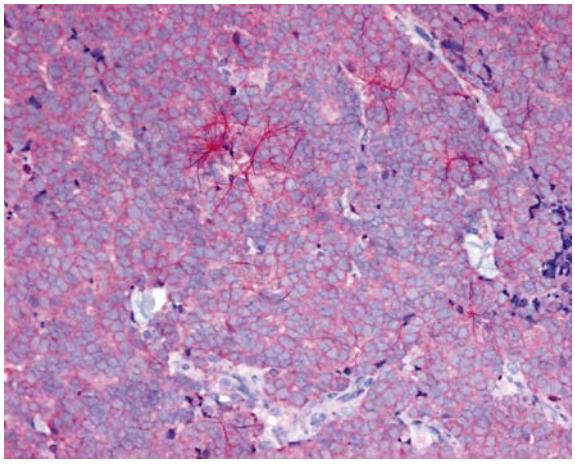

Fig. 19.48 Merkel cell tumor stained with an antibody to neuron-specific enolase using alkaline phosphatase detection with red chromogen, demonstrating 'spider web-like' precipitate across the section.

Automation error

The use of automated platforms has assisted in improving staining quality and consistency but is not foolproof and can create unique artifacts of its own. Depending on the type of instrument used,

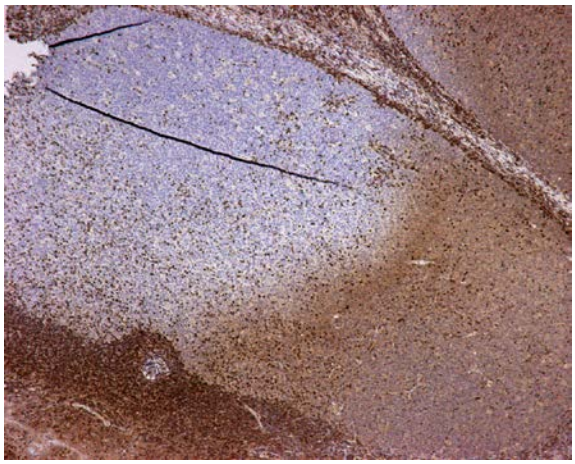

Fig. 19.49 Lymph node stained with an antibody to Bcl-2, demonstrating poor tissue coverage due to air bubbles, and non-specific staining due to poor rinsing.

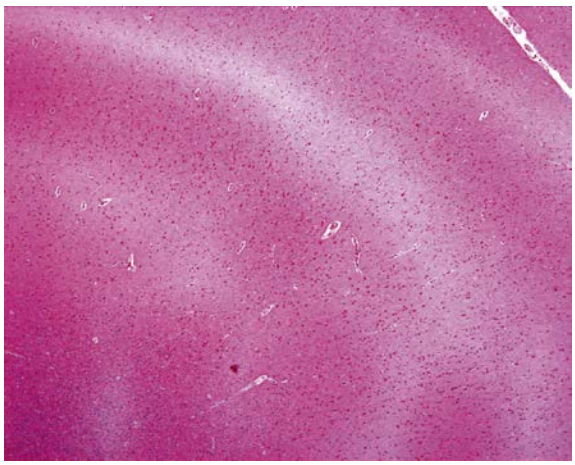

Fig. 19.50 Brain stained with an antibody to polyclonal ubiquitin using alkaline phosphatase detection with red chromogen. Bull's eye pattern created on a horizontal stainer with the blowing head too close to the tissue.

insufficient rinsing can be a problem, this may be amplified during the chromogen application. Horizontal staining instruments require careful leveling on installation, to prevent reagents slipping off the slide and causing incomplete coverage of the sections. A faulty probe can result in empty or partial draws of reagents, resulting in the dispensing of air bubbles onto the slide (Fig. 19.49). In instruments which blow over the top of the tissue to mix reagents, the probe height must be set correctly or it can create a bull's eye pattern of staining (Figs. 19.50 and 19.51).

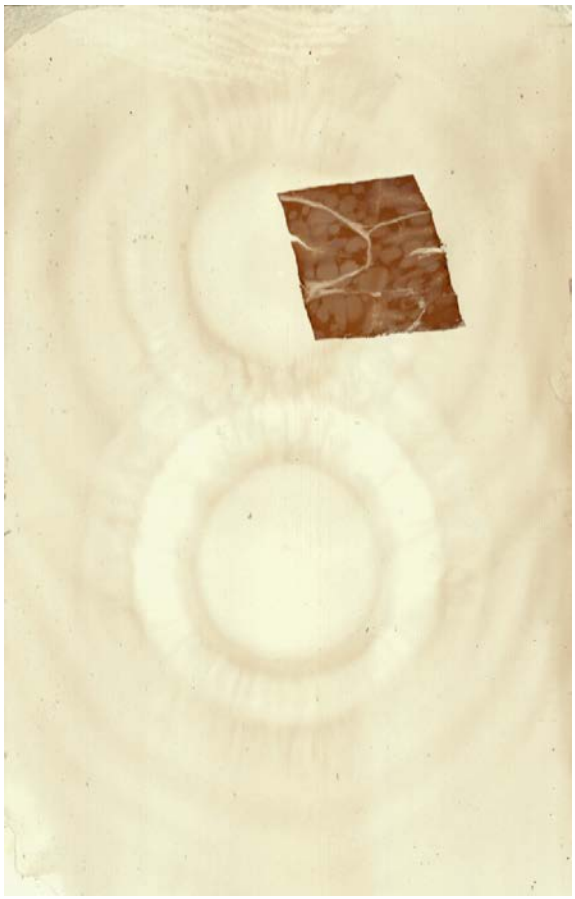

Fig. 19.51 Tonsil stained brown with an antibody to CD3, demonstrating bull's eye artifact on the slide adjacent to the tissue. It is, visible to the naked eye and created by the autostainer's blowing head being too close to the slide.

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20 Molecular pathology

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Introduction

Tumor diagnosis relies centrally on careful morphological examination, supplemented by the judicious use of immunohistochemistry. The information gleaned by these means remains, without doubt, the single most important initial determinant of a patient's prognosis and likely response to treatment. However, in recent years the importance of molecular assessment, as an adjunct to this examination has grown dramatically. This has largely been driven by the emergence of new 'targeted' therapies where certain molecular markers identify subgroups of tumors which may or may not respond to specific drug treatments (Zeron-Medina et al., 2015). Testing for these markers is now mandatory prior to any decision on the type of novel treatment. It has also been shown that a range of molecular markers can contribute useful information for the diagnosis and prognosis of particular tumors.

The evolving field of molecular pathology has produced a great deal of excitement by targeting particular therapies to specific tumors, offering two related sources of hope. Firstly, patients may be provided with more effective treatments improving their quality of life; secondly, ineffective treatments with their concomitant side-effects may not be administered to patients in vain.

The tremendous promise of this new approach also presents substantial challenges.

- There are significant technical difficulties inherent to molecular testing which must be overcome first.
- Newly formed molecular pathology services must be effective and deliver results within reasonable time frames.
- Molecular techniques must fulfil stringent accreditation requirements and must be affordable within public health systems.

Perhaps the greatest challenge presented by molecular pathology is its rapid pace of change. This should be expected for such an emerging and hopeful enterprise, within which new opportunities abound. Against this backdrop, it is easy to lose sight of the fact that the very reason for this endeavor is to contribute clinically relevant information which improves patient diagnosis, management and prognosis.

This principle, on the one hand, supports a degree of parsimony by encouraging, in routine clinical practice, the judicious use of those molecular tests which are likely to contribute actionable insights and so, reinforces the need for strong clinicopathological correlation in molecular assessment. On the other hand, it mandates that practitioners maintain an open mind to molecular assessment and that they adopt whatever approaches and platforms are best suited to delivering clinically useful information. An effective molecular pathological service therefore, ought not to limit tumor profiling to details about specific gene mutations, but should combine this with relevant information about tumor cell chromosomal alterations and protein expression.

The newer developments in this growing field leave even this formidable requirement wanting. For example, it is now known that the assessment of a tumor's immune microenvironment can have substantial clinical implications giving rise to the burgeoning field of immunotherapy. Additionally, it is now possible to assess some molecular alterations indirectly as an adjunct to tissue molecular

assessment by analyzing circulating tumor DNA in plasma. From this, it is not difficult to appreciate how easy it is to be overwhelmed by the extraordinary and increasing amount of information which these various modalities provide. Indeed, the great challenge facing molecular pathology practitioners is to sensibly select from these disparate modalities, based on clear clinical rationales, and to integrate their contributions into coherent and clinically useful reports.

This chapter reviews the various techniques used in molecular pathological assessment with an account of their challenges and limitations. It progresses to discuss how the data from these tests are used to guide patient diagnosis, treatment and prognosis, before finally detailing examples of the use of molecular pathology in some specific common tumors.

To illustrate the different methodologies available for molecular testing, real-life supplier examples are described, but please note that this is not a recommendation and alternative providers and protocols are available.

Glossary of terms and abbreviations commonly used in this chapter

Accreditation
The process of ensuring that an organization or process is able to deliver reliable results.

Amplification
1. Production of additional copies of a gene sequence. This multiplication of the sequence makes it easier to identify.
2. A mutation which results in the repetition of a gene within the DNA sequence, leading to overexpression of the encoded protein.

Anneal
To join two strands of complementary nucleic acid.

Base
A building block of the nucleotide. There are four different bases for DNA, adenine (A), thymine (T), cytosine (C) and guanine (G). In RNA, thymine is replaced by uracil (U).

Base pair (bp)
Two bases which are complementary to each other and joined by hydrogen bonds.

Bioinformatics
A field which seeks to analyze biological data, usually making use of computer science and statistics.

'Black box' system
A molecular testing system which performs all data analysis internally and presents only the final result, e.g. mutation present or not.

CE-IVD (Conformité Européene, in vitro diagnostic)
Indication that a given diagnostic test has reached the required safety and efficacy level mandated by the European Union Directive 98/79/EC. It is required before tests can be marketed in the EU.

Chromosome
The structures into which DNA strands are organized. Humans have 46 in total, comprising 23 pairs.

Codon
A set of three consecutive nucleotides in DNA or mRNA which directs the incorporation of an amino acid during protein synthesis, or signals the start or stop of translation. Multiple codons will code for the same amino acid.

Companion diagnostic
A diagnostic test which determines whether a patient is suitable for a particular treatment.

Complementary sequence
Nucleic acid sequence of bases which can form a double-stranded structure by matching base pairs. For example, the complementary sequence to C-A-T-G (where each letter stands for one of the bases in DNA) is G-T-A-C.

Deletion
The loss of part of the DNA sequence.

Denature
To dissociate a double-stranded region of nucleic acid into the homologous single strands by breaking the hydrogen bonds, usually achieved by heating.

Deoxyribonucleic acid (DNA)
The genetic material in chromosomes.

DNA polymerase
An enzyme which synthesizes new DNA using a parental DNA strand as the template.

Driver mutation
A mutation which drives carcinogenesis. Targeting a driver mutation is likely to have clinical benefit. Compare with *passenger mutation*.

Exon
A part of a gene which is ultimately used to encode a protein. All the exons within a given gene are numbered.

Extraction
The process of removing DNA from a biological sample.

Fluorescence in situ hybridization (FISH)
A cytogenetic technique using fluorescent probes which bind to only those parts of the chromosome with a high degree of sequence complementarity. FISH is often used for finding specific features in DNA for use in genetic counseling, medicine and species identification. It can also be used to detect and localize specific RNA targets (mRNA, long non-coding RNA and microRNA) in cells, circulating tumor cells, and tissue samples.

Food and drug administration (FDA)
The federal agency which is responsible for establishing the safety and efficacy of drugs in the USA.

Gene
A DNA segment which codes for a polypeptide.

Genome
The total amount of DNA present in a cell.

Germline mutation
A mutation which is inherited. Compare with *sporadic mutation*.

Helicases
Enzymes which bind and may even remodel nucleic acid or nucleic acid protein complexes.

Home-brew assay
An assay developed 'in house', in contrast to a commercially available kit.

Hot-spot
An area of the genome which is particularly prone to containing mutations.

Hybridization
The action whereby two complementary single-stranded pieces of nucleic acids are joined to form a double-stranded segment.

Immunohistochemistry (IHC)
The use of labelled antibodies to highlight particular antigens in tissue.

Immunotherapy
The treatment of a disease, often cancer, by modulating the immune system.

In situ
In the normal location, i.e. an in situ tumor is one which is confined to its site of origin and has not invaded neighboring tissue or spread elsewhere in the body.

In situ hybridization (ISH)
A technique which identifies and quantifies nucleic acid sequences within cells.

Insertion
The addition of nucleotides into a DNA sequence.

ISO standards
International standards ensuring that products are safe, reliable and of good quality.

Kilobase (kb)
A measure of the length of nucleic acids. One kb equals 1000 nucleotides of single-stranded nucleic acid; kbp refers to kilobase pairs of double-stranded DNA.

Macrodissection
The process of removing tissue from a slide which is not of interest for testing. In this context, removing non-tumor tissue from a slide for molecular testing.

Melting temperature
The temperature at which the hydrogen bonds between complementary nucleotides will break, causing the dissociation of double-stranded nucleic acid. It is dependent on the G + C content of the DNA.

Messenger RNA (mRNA)
Single-stranded RNA synthesized from a DNA template during transcription which binds to ribosomes to direct protein synthesis. It carries the message of the DNA to the cytoplasm of the cell where protein is made.

Methylation
The process by which methyl groups are added to DNA. When gene promoter regions are hypermethylated, transcription of the genes they control is repressed.

Microsatellite
A series of short, repetitive DNA sequences normally occurring in DNA. They are more likely to accumulate mutations if there are abnormalities of the mismatch repair system.

Mismatch repair (MMR)
This system comprises four proteins and repairs errors incurred during DNA replication. This is important in colorectal cancer.

Missense mutation
A single base substitution in DNA which changes a codon for one amino acid into a codon for a different amino acid.

Multiplex testing
The process of testing for more than one molecular alteration simultaneously.

Mutation
A change in the sequence of nucleotides in DNA.

Nucleic acid (NA)
Large polymers made up of nucleotides which encode genetic information, i.e. DNA and RNA.

Nucleotide
The unit of DNA or RNA which consists of a phosphate group, a sugar and a base.

Oligonucleotide
A short piece of nucleic acid which can be used as a hybridization probe.

Passenger mutation
An incidental mutation which does not drive carcinogenesis. Compare with *driver mutation*.

Plasma
The fluid component of blood which comprises water, proteins and electrolytes, excluding blood cells.

Polymerase chain reaction (PCR)
A technique which amplifies a length of DNA repeatedly, across several orders of magnitude, generating thousands to millions of copies of the particular DNA sequence.

Probe
A single-stranded piece of labeled DNA or RNA which will bind to a complementary sequence, known as the target.

Replication
The process by which an exact copy of parental DNA or RNA is made with the parental molecule serving as a template.

Ribonucleic acid (RNA)
A nucleic acid which plays an important role in protein synthesis and other cell activities.

Sensitivity
The likelihood of developing a mutation when a mutation is genuinely present. A low-sensitivity test is likely to be associated with false negative results.

Single nucleotide variant (SNV)
A mutation involving a single DNA base.

Sporadic mutation
A mutation which occurs randomly, rather than being inherited. Compare with *germline mutation*.

Targeted therapy
A therapy, usually a drug, which is given to certain patients based on a molecular marker.

Template
A strand of DNA or RNA which specifies the base sequence of a newly synthesized complementary strand of DNA or RNA.

Topoisomerases
An enzyme group which participates in the overwinding or underwinding of DNA.

Transcription
The process by which a DNA sequence is copied into a complementary RNA sequence. An RNA transcript is the RNA sequence which results from transcription of a particular sequence of DNA.

Transfer RNA (tRNA)
Small RNA molecules which carry amino acids to the ribosome for polymerization into a polypeptide.

Translation
The process by which mRNA is read, to produce a protein.

Translocation
The process by which part of one chromosome is transferred to another chromosome.

Tyrosine kinase inhibitor (TKI)
A class of drug which inhibits tyrosine kinases, e.g. epidermal growth factor receptor (EGFR).

Validation
The process of acquiring evidence that a procedure will consistently generate accurate results. In context, ensuring that a molecular test genuinely predicts treatment response or prognosis.

Wild-type
The 'normal' form of a gene, i.e. not mutated.

Essentials of molecular pathology: nucleic acid structure and function

To fully understand how the manipulation of genetic material in the laboratory is essential in molecular pathology, the fundamental properties of DNA and RNA must be appreciated. DNA is the inheritable template of reproductive information from which all cellular processes begin. Careful regulation, reproduction and rearrangement of DNA function forms the basis of healthy biological pathways and most pathological conditions originate from the disruption of these mechanisms.

From what is DNA made?

Nucleotide bases, formed from variants of deoxyribose, linked by a phosphate backbone, are the macromolecular units of DNA and RNA. DNA typically exists as a double-stranded, 'right-handed' helix (the structure twists to the right along its length). Within helices, adenine (A) and guanine (G), the purine bases, pair respectively with thymine (T) and cytosine (C), the pyrimidine bases. This relationship, known as 'complementarity' is essential to the replication and hybridization techniques used in the laboratory. RNA is a single-stranded structure which 'self-anneals' to produce a three-dimensional shape and the base thymine (T) in DNA is replaced by uracil (U) in RNA strands.

The double helix in which DNA exists in most physiological conditions is held together by double (A-T pairs) or triple (G-C pairs) hydrogen bonds which can be overcome through heating or exposure to alkaline pH, 'denaturing' the structure. The temperature required to induce denaturation of the triple hydrogen bonds present between G-C pairs is directly related to the

proportion of these groups, the so-called 'GC content'. When the environmental temperature is reduced again, the bonds between strands reform to restore the double stranded structure, a process known as annealing. Annealing occurs only between complementary strands and thus knowledge of a target sequence's structure helps the scientist determine the specific conditions necessary to denature or hybridize DNA strands and to discriminate between different sequences present within the DNA, forming the technical basis of PCR or ISH techniques.

How does DNA replicate?

The division of cells to form growing organisms, renew and repair existing tissues, or produce haploid cells (containing single copies of each chromosome, e.g. during reproduction) depends upon the ability of cells to replicate their DNA. Cellular enzymes act in cohorts to separate double stranded DNA, initiate extension of new complementary strands through the addition of new bases and finally, reform the DNA helix to produce two new copies of the DNA from each existing strand (Fig. 20.1). As topoisomerase and helicase advance along the helix, they 'unwind' and separate strands to leave them exposed for the addition of complementary bases. This is essentially the process which we can reproduce using temperature controlled denaturation in the laboratory. DNA polymerases then follow, adding complementary bases which will form the new, opposing DNA strand. In the laboratory, the power of polymerases is harnessed to reproduce DNA and amplify many copies of our target sequence for interrogation.

From blueprint to building blocks: transcription and translation

Human DNA provides a hereditable, reproducible 'plan' which the cell can draw on to construct its protein components. This takes the form of a multi-stage process in which DNA is first *transcribed* into single-stranded RNA and subsequently *translated* into amino acids and, in turn, protein macromolecules. The initiating phase relies upon enzymatic separation of DNA to produce single strands; transcription then utilizes an RNA polymerase which engages with a so-called 'start sequence' or promoter region in single-stranded DNA. This specifically signals that the subsequent bases within the DNA are to be transcribed. The polymerase introduces complementary G, C, A and U bases until the enzyme reaches a 'stop' region, signaling that RNA transcription must cease. The complete, single-stranded RNA molecule is then ready for export out of the nucleus into the cytoplasm where a variety of post-transcriptional modifications render RNAs suitable for an array of different roles. Vital to protein synthesis, it is mRNA which acts as a template for protein molecule synthesis by the cell. Template mRNA functions by forming

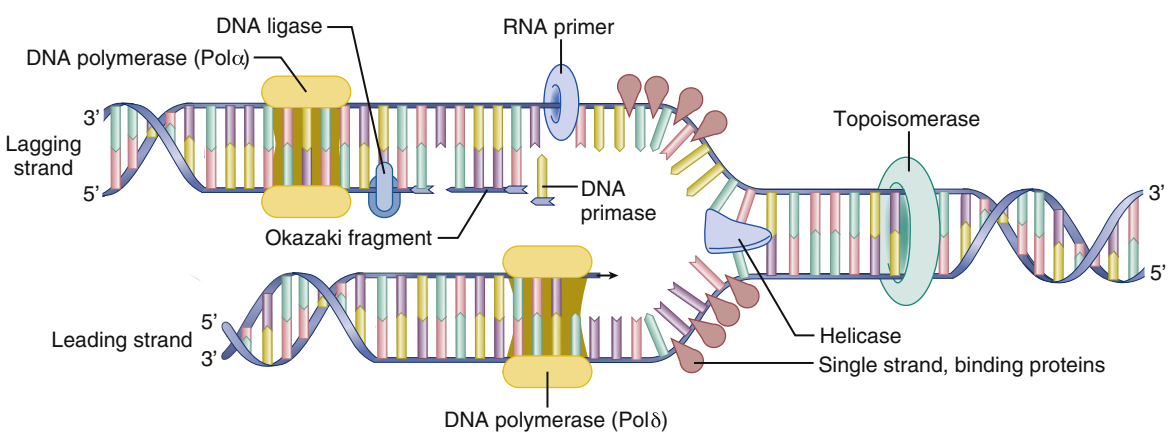


Fig. 20.1 The replication of DNA, showing the various enzyme sites of action which allow duplication of a DNA chain (redrawn with permission from https://pixabay.com/en/diagram-dna-biology-labeled-41531/.)

three-base units of genetic code known as codons. Each individual mRNA codon has a complementary anti-codon on a second form of RNA molecule, tRNA. The transfer RNAs each code for a particular amino acid, however, considering that the 4 different RNA bases give a total of 64 different possible combinations, and only 20 different amino acids are produced by translation, there is some 'redundancy in coding'. For example, the codons UUU and UUC both encode the amino acid phenylalanine, whilst UUA and UUG translate to leucine. Transcription and translation allow the specific, regulated, reproducible production of amino acid sequences directly governed by the DNA sequence. With this relationship in mind, the significant impact of even a single base alteration to DNA sequences, which alter the final translated protein product, producing a pathogenic variant is obvious.

Techniques in molecular pathology

The current range of molecular pathological techniques is impressive and it is easy to be overwhelmed. A helpful way to consider molecular pathology techniques therefore, is to think of them in terms of the structure of the gene and its relationship to gene expression. *Individual genes* are located on *chromosomes* and their transcription gives rise to *RNA*, whose translation leads to *protein expression*. Tests can then be divided into those which examine:

1. Mutations in *individual genes*, the polymerase chain reaction (PCR).
2. Abnormalities of overall *chromosome structure*, predominantly fluorescence in situ hybridization (FISH).
3. Abnormalities in *RNA*, in situ hybridization (ISH).
4. Changes in *protein expression*, immunohistochemistry (IHC).

There are two further types of molecular testing techniques which do not fit so neatly into this scheme:

5. In some circumstances, it can be difficult or impractical to examine these factors directly and in some of these cases, where validated tests exist and clinical requirements are satisfied, it is common to use more easily assessable *surrogate markers* for alterations. An example, discussed in more detail later in this chapter, is ALK translocation testing where the presence of a translocation can be examined directly using FISH, but it is now more common to use IHC to examine for secondary ALK protein expression abnormalities.
6. *Multiplex testing* modalities allow multiple molecular targets to be assessed in a single specimen, a situation which is becoming increasingly common as the number of actionable, molecular alterations increases. One example is next-generation sequencing, a highly sensitive method of examining for multiple genetic alterations which is described in more detail later in this chapter.

Before considering any of these specific modalities, it is absolutely essential to have a solid grounding in the basic processes of tissue handling and processing which are common to all of these techniques. The success or failure of any of the above techniques relies entirely on good understanding and implementation of these processes.

Practical considerations for the laboratory scientist

Practicalities of tissue sample workflow

Modern laboratories are moving towards increasing efficiency, often in the form of 'LEAN' working (see Chapter 1), whilst conversely adopting greater levels of monitoring and documentation in order to meet financial restrictions and accreditation requirements. Molecular pathology practices require tailoring of processes throughout the workflow to optimize delivery of specialized analyses, and these must conform to the overall structuring of both laboratory practice and administration. Scientists should consider their responsibility for:

- Optimization of pre-analytical workflows to support downstream analysis.
- Minimizing specimen 'wastage' for the reasons detailed above.
- The precise monitoring of specimen handling parameters and metrics.
- Delivery of relevant data to the clinical 'end user' which answers the question being asked.

Fundamental factors in pre-analytical workflows

- *Pre-fixation and specimen transport times.* Excision of human tissue immediately triggers a cascade of changes to molecular expression. Whilst pathology laboratories and diagnosticians are hugely experienced in overcoming these changes using more traditional methods and analyses, the impact upon molecular pathology techniques is less well known. Hypoxic times, for example, directly alter 'molecular' targets such as RNA expression as well as 'traditional' variables like mitotic figure counts (Cross et al., 1990).
- *Fixation, typically using formaldehyde.* As discussed in greater detail later, formaldehyde, typically as NBF, is a 'mixed blessing' as the cross-linking which stabilizes and preserves tissue, also fragments DNA. Fragmentation limits the maximum length of DNA regions which can be targeted and amplified in the PCR reaction, the 'amplicon'. Highly fragmented DNA is non-amplifiable, and the rate of PCR failure correlates directly with the severity of fragmentation. Repeatability and reproducibility of molecular analyses cannot be guaranteed without adequate processing controls which include monitoring of fixation times. Both minimum (Qizilbash, 1982) and maximum (Nam et al., 2014) fixation times should be regulated, with consideration as to whether durations greater than 24hrs are necessary.
- *Physico-chemical influences.* The temperature and chemical components of the processing pathway both affect biomolecule stability and these must therefore be strictly regulated. Changes to processing times or methods, including 'novel' fixatives, or processes such as the use of high temperatures and/or microwave radiation, require thorough validation. The effect upon molecular pathology analyses of 'traditional' histopathological techniques such as decalcification must be fully evaluated and understood.

Optimizing the use of tissue

Modern diagnostic histopathological processing relies upon high throughput with streamlined generic protocols allowing clinical demands to be met. However, these can lead to wastage of material as excess tissue is readily lost during preparation of sections. Scientists can play a key role in balancing the need for rapid delivery with the most efficient use of limited available material. Considerations include:

- Retention of intervening sections between preparation of 'levels' for subsequent IHC or molecular analysis.
- Preparation of additional, specifically tailored tissue sections during initial diagnostic work, for later use in 'standard of care molecular diagnostic work' (see below).
- Rationalization of current diagnostic testing, e.g. the Royal College of Pathologists 2014 Dataset for lung cancer histopathology reports (Nicholson et al., 2016).

Processing the sample

The hands-on processing of samples can be broken down into the following basic steps.

Specimen preparation and assessment

Whilst this varies widely depending upon local laboratory preferences, formalin fixed paraffin wax embedded (FFPE) tissue is usually made into sections (mounted or unmounted) of tissue cores. Cytological fluids are increasingly being used for molecular testing, they may be centrifuged to form cell pellets or precipitates which are then embedded in paraffin wax and analyzed. Increasingly, even after initial final diagnosis is complete, new H&E-stained sections are prepared to confirm the tumor population remains present and help review the technique used.

Extraction and/or isolation of target molecules

DNA analysis, barring in situ techniques, almost inevitably involves a degree of specimen homogenization. Scientists must consider whether dilution of the target population amongst 'bystander' normal cells, which are unlikely to carry the alteration, being tested (whatever the biomolecule), may render it beyond the limit of detection for a given assay. In this case, should greater sensitivity not be possible or if it could create further issues, tissue macrodissection is an option. This allows, to some extent,

the exclusion of other unwanted tissue components such as pigment and necrosis, although studies suggest that high levels (>50%) of tumor necrosis are not a significant source of analytical variability if a sensitive detection method is employed (Fisher et al., 2014).

In simple terms, macrodissection is the physical removal of target tissue from a section/slide and exclusion of unwanted areas. Often this is as simple as scraping tissue from slides with a scalpel or pipette tip and transferring it to a new receptacle. Removal of wax and/or rehydration is then needed as in traditional histopathological techniques. This may be through common xylene-alcohol-water transition processes or via the use of more complex proprietary deparaffinization solutions.

Amplification and measurement

The amplification of nucleic acids can, in itself, form the basis of an analysis, the 'direct' PCR methods, which primarily assess the presence or absence of a sequence. It more commonly provides an initial step which affords the investigator with ample target to analyze. Unamplified nucleic acid sequences may be too scarce to thoroughly investigate. As a precursor, PCR amplification may then be followed by 'reading' of the nucleotide product, classical 'Sanger' sequencing or pyrosequencing or 'measurement' of the quantities amplified. Examples of this are quantitative, real-time and allele-specific PCR which are discussed in further detail later.

Analysis and interpretation

Once the data are available they need to be interpreted. Sequence data, such as the DNA sequence information derived by performing Sanger sequencing, can be interpreted by comparison to known, curated sequence data for a given organism, chromosome or gene. The interpreter can access such data via resources such as the National Center for Biotechnology Information's (NCBI) Entrez Pubmed database or the Wellcome Trust Sanger Institute's Catalogue of Somatic Mutations in Cancer (COSMIC). Data relying on measurement of the level of the product, or rate of production, are instead usually analyzed quantitatively against established reference templates or standards to determine levels of background 'noise' and significance.

Reporting of findings

Analysis and reporting requires interpretation in context which is dependent upon the individual specimen and assay employed. Interpretation of data depends upon both scientific knowledge and practical experience to guide judgment. Clinical context allows the reporter to determine the prognostic and predictive significance of a given alteration in different types of malignancies and this is discussed in more detail on page 425.

Standardization and accreditation

The recent transition in the UK from Clinical Pathology Accreditation (CPA) to the international standard for Medical Laboratories, ISO 15189:2012 accreditation under the governance of the United Kingdom Accreditation Service (UKAS) has had a profound impact on the manner in which scientists and medical staff implement, deliver and regulate testing (Long-Mira et al., 2015). The ISO 15189:2012 standard is multifaceted and too complex to consider in depth here; an overview can be found at the UKAS website (see Useful websites) and the full set of standards themselves may be purchased by laboratories intending to align themselves appropriately. In simple terms scientists should consider the measurement, regulation, recording and control of each and every variable within a process, and have attempted to identify and understand the impact of any variable beyond their control. In implementing a new test, the laboratory needs to demonstrate that appropriate validation or verification of this method has occurred. For example, if a method states that any stage within a process should take place for a given period of time at a specific temperature, the laboratory must determine that these requirements are precisely met by:

- Measuring them for the duration using a calibrated reference.
- Recording them appropriately.
- Setting in place measures to re-take, should deviation occur.

- Appreciating the effect of measurement uncertainty or other external, uncontrolled variables, e.g. environmental conditions and user-to-user variation.

Each process should have a constantly updated training plan and clear set of assessable competencies tailored to each of the various staff members involved. For example, the training and competency for a laboratory scientist preparing an analytical reaction would not be identical to that of a scientist interpreting and reporting on those analyses.

Use of control material

'Controls' refer to any analyte or material of known properties which allow standardization or measurement of the analytical performance of an assay. This may be something as simple as sterile water added instead of DNA to a PCR reaction as a negative control, demonstrating that any reactivity/positivity in an assay results only from the presence of the specimen or sample being tested, eliminating the possibility of 'false positives'. Positive controls establish the opposite, by always producing positivity they confirm that the lack of reactivity when testing a patient sample is due to the absence of the target molecule and not because of some inhibition to the reaction, or a technical failure due to operator, reagent or system failure or sub-optimal performance. Positive controls may take the form of a specific variant to demonstrate specificity, or more often where an assay is used to analyze unknown numbers or complexities of variants within a region, a universally amplifiable or detectable sequence such as a 'normal' DNA sequence synthesized from an established region of the genome. ISO 15189 standardization has an impact upon the use of control material just as with any analytical method and a laboratory must demonstrate the adequate control and regulation of a material which is appropriate and comparable for use as a control in assays analyzing human specimens. An increasing number of commercial companies now offer CE-IVD certified control material which laboratories need only verify for use in their assay before implementation (see below and Useful websites).

Techniques testing for mutations in genes: the polymerase chain reaction (PCR)

This is the most basic form of molecular testing where the modalities interrogate the sequence of bases in DNA to identify alterations. These alterations encompass both substitutions of single bases, known as single nucleotide variants (SNVs) and more complex genetic abnormalities such as gene deletions and insertions.

There are several variants of this technique, but the common principle is that primers complementary to particular sequences of interest are added to the DNA sample. Nucleotides and DNA polymerase are added and the whole solution undergoes cycles of heating and cooling. Replication of the original sequence of interest occurs with each cycle and there is an exponential increase in the number of strands of DNA in the solution. As discussed in detail later, different PCR techniques employ different modalities for determining the sequence of the amplified DNA sequence.

Stages of PCR based analysis

Nucleic acid (NA) extraction

Extraction of DNA (and to a lesser extent RNA, given the smaller number of common applications) from FFPE tissue has transformed radically in the past decade. The changes include:

- A proliferation of molecular pathology tests essential, perhaps mandatory, for patient management.
- New commercially available products designed specifically to enhance NA retrieval from FFPE tissue.
- The desire to derive purer, less fragmented NAs which will improve analytical success rates and allow the application of more demanding tests.
- The need to 'do more for less' with relatively smaller specimens.

At the turn of the century many laboratories still employed simple 'home brew' or 'laboratory developed test' (LDT) type extraction methods which were often based on chemical lysis techniques, e.g. the classical phenol:chloroform phase separation (Sengüven et al., 2014). These were more hazardous

and unpleasant for the practitioner and yielded a crude NA isolate of relative impurity. Whilst classical Sanger sequencing was the mainstay of analytical processes, this represented less of an obstacle but, with the increasing adoption of more sensitive qualitative assays, a better alternative was needed.

Many commercial companies had already addressed the issue of NA retrieval from low volume/quality samples in their research, e.g. in low cell population proliferation assays, small volume animal samples or in forensic settings where NAs were often poorly preserved. Laboratories made initial progress with FFPE samples by exploiting these kits.

There have been developments to the main approaches to extraction. The advantages and limitations, and how laboratories can best integrate them in to their overall specimen workflow are discussed shortly. There are no strict or even informal guidelines on how NA extraction should be performed, it is very much determined at the local level on an 'as-needed' basis to meet the requirements of the assays employed by the laboratory.

However, this arrangement may be changing in response to various pressures. As laboratories move towards greater standardization, e.g. in order to obtain ISO 15189 accreditation, commercial kits offer a simpler alternative to self-regulated methods. The increasing adoption of commercial CE-IVD assays ensures that the standard which must be met in order to commercially provide clinical devices or tests within the European Union often comes with specified pre-analytical processes. A particular assay may require in-house validation if that laboratory does not use the manufacturer's specified extraction protocol. NHS England, in association with Genomics England (GEL) has, for the first time, provided some guidance, albeit in a limited form. These guidelines for specimen processing within the 100,000 Genomes project includes detailed protocols on how participating laboratories should extract DNA for submission to its central biorepository and sequencing laboratory (see Useful websites).

Spin column purification and 'on-membrane' DNA isolation

Many commercial manufacturers offer a variation of this now long-established extraction technique. Digested cellular material in suspension is passed over a membrane to isolate free NAs from solution. Subsequent chemical 'washing' removes other cellular constituents before a final elution stage releases purified NAs into a buffer or sterile water solution. Both automated and manual methods are available. For an in-depth explanation of automated systems, such as the Qiagen Qiacube® see Useful websites.

Briefly, a tissue isolate deparaffinized on the section prior to removal and transfer to a sample tube, or deparaffinized in the sample tube itself, is mixed with key components to the tissue digestion process. These are a cell membrane disrupting agent such as a detergent, i.e. a buffer which provides a mildly alkaline pH ensuring acidic degradation and depurination of nucleic acids is inhibited, and a proteolytic enzyme, typically proteinase K. Together, these degrade cellular constituents which would otherwise render nucleic acids inaccessible and allow them to pass freely into solution. This process is performed at above ambient temperature, approximately 56°C, to enhance enzymatic activity and accelerate digestion. It can be further enhanced by agitation to promote mixing and physical disruption. The complete digestion of tissue is heavily dependent upon the starting volumes used and laboratories should tailor protocols to standardize input for optimization of the process. Incompletely digested constituents can severely impair the downstream membrane isolation stage. Addition of buffer, to reduce saturation, and proteinase K, to aid proteolysis, can both be used mid-process to promote more rapid completion.

Once fully digested, the suspension is usually subjected to a short high temperature incubation phase at ≤90°C. Whilst historically this acted to deactivate proteinase K and prevent proteolytic activity in the downstream PCR reaction, it is now understood to assist in formalin-protein crosslinking reversal and enhance NA retrieval. The entire digest is then transferred to the spin column and

centrifuged to isolate cell constituents on the column membrane. If large quantities of digested material are to be used, this process can usually be repeated until all the suspension has been passed through the column. The unwanted solution is discarded and 'clean-up' of the NA performed by one or more 'washing' stages. A wash buffer, typically containing a guanidinium salt such as guanidinium thiocyanate is passed over the membrane by further centrifugation to remove other constituents, mainly proteins, but leave the NA in situ on the membrane. Once complete a dedicated elution buffer, or simple sterile water, depending upon the user preference, is used to release the NA into solution and elution into a sterile dedicated specimen tube for storage and further use.

An adapted version of the standard Qiagen method for FFPE extraction (see Useful websites) was endorsed by GEL as an approved process for retrieving DNA of sufficient quality to be utilized in downstream DNA-'hungry' Whole Genome Sequencing. Spin column methods are versatile and scalable, allowing for fluctuations in throughput without compromising delivery, by requiring batching to remain cost efficient. Where automation is possible, many of the same kit components can be used in both manual and mechanical processes, allowing flexibility of application and continuity of service during machine downtime. However, automation is more liable to loss of NAs during processing when compared to other popular methods, but manual processing is labor intensive, repetitive and more prone to human error.

Magnetic bead isolation

Nucleic acids are inherently charged due to their phosphate backbone and this property can be easily exploited to assist in isolation. By following an identical initial processing pathway to that described above, a crude tissue lysate can be prepared. Instead of opting to perform multiple fluid transfers with associated centrifugation the NA itself can be transferred from solution to solution, achieving the same process. Magnetically charged beads are added to the lysate and the DNA present will rapidly bind to these. A magnetic rod is then immersed into the solution and the DNA-bead complexes in turn bind to the rod. Risk of cross-contamination and NA carry-over is reduced by employing thin disposable rod covers. These prevent direct binding of DNA to rods but do not interfere with magnetic interactions to the extent that valuable material is not lost. Once the isolation-purification process is complete the rod covers are detached from the rod and rapidly agitated within a specimen tube containing suspension fluid, allowing the DNA-bead complexes to become free from any attraction and released into suspension.

Bar the initial addition of prepared tissue lysate and any post-processing (see below), minimizing pipetting in the processing chain reduces the risk of cross contamination and carry-over of material when compared to spin-column type methods. The loss of liquid and material by residual binding to pipette tips is no longer a concern. The final DNA suspension is not absolutely ready for use since the magnetic beads used for isolation remain present and can cause interference with the molecular application. However, they are relatively easily removed in a subsequent precipitation to leave pure DNA. Although these methods are not entirely without some manual processing stages they usually employ sealed, single use, 'per-sample' kits containing all the necessary reagents in discrete compartments. As such, without the automated system to process them, they are entirely redundant, and unlike many spin-column methods they cannot be hand-processed as a 'backup' method should equipment failure occur. To ensure continuity of service a laboratory using magnetic bead isolation will need to maintain and support an alternative method if downtime is to be avoided during service or technical failure.

Ultrasonication methods

Although perhaps less widespread than the methods above, ultrasonication is worthy of consideration for some of its novel features and advantages. The theoretical basis of the method is that exposure of a liquid medium to ultrasonic waves will agitate and disrupt cells or tissues within it. Practically, tissue, often in the form of cores or fragments rather

than sections, is immersed in buffer and subjected to bursts of ultrasound which dissociate the tissue from the paraffin wax and rehydrate it. Subsequent proteinase digestion, cross-linkage reversal and purification is carried out similarly to the spin column methods. Advantages include:

- The use of an aqueous medium, eliminating the use of organic solvents and other hazardous chemicals.
- One-step single stage dissociation of paraffin wax and tissue, including tissue disruption.
- Larger volumes of starting material can be used as larger volumes of paraffin wax, which are slow to dissolve chemically and easily block spin-columns if not fully disrupted, are easily rendered into emulsion by ultrasonication.

This capacity to increase tissue input and resulting DNA output, along with a higher yield of DNA fragments from across the genome, make this form of extraction popular with some users of 'DNA-hungry' next generation sequencing (NGS) methods.

PCR analysis methods

Manipulating the amplified DNA (our PCR 'products') post-PCR, or as a 'real-time' process during the PCR itself, allows one to obtain qualitative and quantitative data. A wide variety of PCR variants exist which vary the fundamental cycling parameters previously discussed to different effects.

- 'Cold' PCR exploits the subtle differences in melting and annealing temperatures which are caused by small variations in the sequence of a given region. By identifying these and adjusting accordingly, the PCR reaction can be 'biased' to favor amplification of a mutant sequence over the 'wild-type' variant and allow variants present at very low levels to be more easily identified.
- 'Touchdown' PCR employs a graduated decrease in the annealing temperature of each cycle, usually descending incrementally for around 10 cycles, before settling at a low annealing temperature for the remaining 20-30 cycles. The lower temperature employed for the later cycles means that primer sets with different annealing temperatures can be amplified simultaneously and a dedicated thermocycler is not needed for each protocol. However, the initial higher annealing temperatures ensure that the first few rounds of amplification are specific, and the later lower temperature does not lead to non-specific amplification as expected because multiple copies of the specific product are already amplified, favoring their reproduction.

Below, we consider a few of the fundamental PCR-analysis methods.

Sanger sequencing

The Sanger method has been a mainstay of laboratories for more than 30 years and has only recently begun to lose its status as the 'gold standard' of analysis. It remains the technique of choice in cases where mutations are not limited to hot-spots and where sequencing a whole length of DNA is the only means of identifying relevant mutations. Amplified PCR products are subject to a second round of PCR over a shorter series of cycles, utilizing a series of fluorescently tagged dideoxy-nucleotides (ddNTP) which terminate chain extension in the reaction when incorporated. These 'labelled' products are then subjected to a purification process where excess fluorescent tags and nucleotides are removed, a process which is vital in ensuring that background 'noise' is reduced and a more specific sequence is given. Finally, labelled products are run through a polymer-filled capillary and subject to electrophoresis to separate them based on size. Since the chain is terminated by ddNTP inclusion, both the type of nucleotide incorporated, ddATP, ddGTP, ddCTP or ddTTP, are each labelled with a different fluorophore. The length of fragment can then be determined and the nucleotide present at each position of the chain defined.

Real-time PCR

This variant involves PCR with subsequent quantification or analysis of PCR products and usually employs fluorescent probes or DNA-philic molecules. The amount of fluorescence emitted equates to the amount of PCR product and allows partial quantitation. The amount of PCR product measured

after each PCR cycle is used to generate a plot of PCR product against time. Real-time PCR developed from a desire to try to quantify the products of amplification and the realization that 'end-point' PCR was not always accurate enough to be fit for purpose. End-point PCR relies upon running a PCR to its completion, then performing a measurement of the total product, e.g. by fluorimetry or gel-based methods. However, variables such as the starting template quality, quantity and length, and the reaction efficiency itself easily skew this measurement. Real-time PCR partially eliminates the impact of these factors.

Classic examples of real-time PCR applications usually involve quantitation of products. This could involve measuring levels of a gene of interest versus a control gene, i.e. expression; assessing an increase or decrease in the levels of a given variant, e.g. monitoring of disease response or progress; and calculating the copy number of an agent, i.e. viral load. The high degree of sensitivity the technique provides makes it highly attractive and this can make it routinely employable where low overall quantity or quality of the starting template means that other 'visualization' techniques may fail. The technique is best suited to analysis of restricted region point mutations, small deletions and insertions, and methylation. Larger insertion and deletions (indels) or genes and regions where mutations are randomly distributed over a wide area, are more difficult to assess because of the number of reactions which would be needed to assess longer regions of DNA.

Most real-time PCR methods can be said to fall under one of the following two strategies, although variations in the format of reagents and conditions can be used to enhance specificity or sensitivity:

1. *Intercalating assays* employ a DNA-philic molecule added to the PCR mix which fluoresces when bound to DNA. As the quantity of PCR product increases exponentially so does the level of fluorescence. Although simple, usually relatively cheap and theoretically applicable to any existing PCR assay, these agents are relatively unselective as non-specific amplification products and primer dimers will also contribute to fluorescence levels and decrease accuracy.
2. *Molecular beacons and 'Taqman' probes* are primer-like sequences tagged with both a 'fluorophore', which emits light, and a 'quencher', which blocks this fluorescence when in close proximity. The probe binds to a target sequence lying between the two standard PCR primers and when amplification occurs, extension from the primer physically lyses the probe, the fluorophore and quencher are released, and the former begins to emit light. Each round of amplification increases overall levels of fluorescence and because the emission is strictly linked to amplification of the targeted region it is much more specific than intercalation. In order to design your probe however, you must first know the sequence at your region of interest and this synthesis is relatively expensive, particularly the fluorescent dyes.

Pyrosequencing

This two-stage process involves investigation of relatively short sequences, typically no more than 30 nucleotides in length, in real-time, through the production of fluorescence each time a nucleotide is incorporated into the extended strand during the second round PCR. In the first round, either forward or reverse primers are 'tagged' with a biotin molecule. Incorporation into the amplified product means that all copies of either the forward or reverse strand are highly attracted to streptavidin. By localizing biotin-tagged DNA to streptavidin-coated beads in solution, precipitation with ethanol, and subsequent denaturation of double-stranded DNA with sodium hydroxide releases the untagged strands. Free, untagged DNA can be washed away and tagged, and unidirectional DNA is physically removed from solution by passing over filters through vacuum action. Isolated strands are then subject to a second round of amplification using a 'sequencing' primer which anneals near or immediately proximal to the region of interest. This sequencing PCR is performed in the presence of ATP sulfurylase, luciferase and apyrase and, unlike other forms of PCR, each individual nucleotide is added to the mix individually in turn, rather than a mix of all four nucleotides as in Sanger sequencing. When a given nucleotide

is incorporated, the phosphate released is used to produce ATP by ATP sulfurylase and the ATP leads to the emission of light by the action of luciferase. The apyrase 'deactivates' free phosphate or nucleotides, so emission is brief and specific only to those instances when nucleotides are added. Monitoring which nucleotide has been added, and at what point in the reaction fluorescence is detected, determines exactly which nucleotide is present at a given position, and what proportion is a particular base, e.g. where one of two strands in the DNA contains a variant.

Pyrosequencing is known as a 'sequence-by-synthesis' method. Unlike 'end point' methods in which a PCR product is measured after amplification is complete, addition of each nucleotide during primer extension is quantitatively assessed in real-time. This gives pyrosequencing one advantage over real-time PCR. Although less sensitive, prior knowledge of the variant being sought is not needed, and different variants within the short region amplified can be precisely assessed. In terms of sensitivity, pyrosequencing is better than Sanger sequencing and is considered able to detect variants present at around 5% of the total population. It also requires shorter processing times to complete, although overall coverage is reduced by the need to assess shorter amplicons. The fragility of biotin tags requires careful handling, with loss of sensitivity if primers are repeatedly freeze/thawed over many cycles. Expertise in physical handling of the isolated products is essential to ensure standardized results. Pyrosequencing technology is patented by the biomolecular product specialist Qiagen (see website).

Approaches to PCR testing

In addition to classification by the technical process by which sequencing is achieved, these systems can, more practically, be divided into off-shelf and black-box systems. Off-shelf systems require a degree of interpretation of the results provided by the kits and include Qiagen Rotor-Gene Q®. By contrast, black-box systems perform the entire analysis and data interpretation themselves, presenting the user only with the final results; examples are Roche Cobas® and Biocartis Idylla®, the latter system also performing DNA extraction itself.

Unlike Sanger sequencing, these platforms, accommodating validated kits (CE-IVD and FDA approved), are highly sensitive (as low as 1-3%) while still being highly specific. Consequently, they are more likely to deliver reliable results when small tissue samples are used.

A development which promises to improve the sensitivity of gene sequence testing soon is digital PCR. By splitting the sample into multiple reaction chambers operating in parallel, this technique is able to detect mutations in smaller tissue samples. Various platforms are already available and include Droplet Digital PCR and BEAMing® technology.

Uses of PCR

PCR is an extremely powerful technique which has adopted central importance in molecular pathology laboratories because of its versatility in testing for an exceptionally broad range of genetic abnormalities. The advent of targeted therapies for certain cancers has immensely increased the importance of PCR-based technologies.

Pharmaceutical companies develop targeted drugs alongside a *companion diagnostic*. The companion diagnostic is an assay which identifies a limited number of alterations in specific genes shown in trials to predict response to the drug in question. Even within the same gene, different diagnostics identify different mutations which have been validated for that specific drug. Indeed, using a different assay to that employed in trials for a particular drug can generate confusion because it may identify mutations for which the drug has not specifically been tested.

EGFR1 in non-small cell lung cancer (NSCLC)

It has been demonstrated that a proportion of NSCLCs bear activating mutations in the EGFR1 gene which lead to uncontrolled cell proliferation and tumor development. A number of specific mutations in EGFR1 have been associated with good and sometimes dramatic responses to tyrosine kinase inhibitors (TKIs). There are currently three TKIs

which are licensed for EGFR1-mutated NSCLC. Only a limited number of mutations have been clinically validated and all are located in hot-spots distributed across exons 18 to 21. It is therefore necessary for assays examining for EGFR1 mutations to assess this broad length of DNA for a range of different mutations.

The issue of compound diagnostic drug matching is particularly relevant in the case of EGFR1 mutations in NSCLC. The drug from Astra Zeneca has been validated with the Therascreen kit and the drug from Roche has been validated with the COBAS kit. Both assays target approximately 30 mutations within the four exons of interest, but without complete overlap. Not all possible changes found in tumors are covered by either of the two assays. If direct sequencing is performed in tumors, 13% more mutations will be found than the assays would detect. However, the clinical significance of those extra mutations has not been demonstrated and so testing for them in routine clinical practice is unlikely to be of help.

BRAF in melanoma

It has been shown that mutations in the BRAF gene are associated with good responses to anti-BRAF therapy in melanoma. Two drugs are currently licensed for this purpose, one from Roche and the other from Boehringer. Amongst all the mutations which can possibly occur in BRAF, only a limited number have been shown to have clinical value, i.e. are clinically validated. PCR techniques targeted specifically against these mutation hot-spots must demonstrate the presence of a mutation before anti-BRAF therapy can be commenced.

KRAS and NRAS in colorectal cancer

It is known that a proportion of patients with advanced colorectal cancer will respond to treatment with anti-EGFR1 monoclonal antibodies and two such drugs are currently available for this purpose. However, it has been shown that the presence of either a KRAS or NRAS mutation in the tumor reliably predicts failure of response to these drugs and so testing for mutations in these genes is mandatory prior to prescription. In this way, it is hoped that patients will be spared treatment with drugs which have no beneficial effect.

KIT and platelet derived growth factor alpha (PDGFRA) in gastrointestinal stromal tumors (GISTs)

GISTs are somewhat different to those tumors previously discussed but TKIs (e.g. imatinib and sunitinib) can be highly effective in their management (Jakhetiya et al., 2016). Licensing of these drugs was given more than a decade ago and their prescription relied only on the demonstration of c-kit protein overexpression as demonstrated by immunohistochemistry. Although not mandatory prior to the prescription of TKIs, it is now routine practice to test GISTs for mutations in KIT and PDGFRA before the commencement of treatment. The rationale for this is twofold. Firstly some 15% of GISTs lack mutations in KIT and PDGFA and so would not respond to TKI therapy and secondly a specific missense mutation in exon 18 of PDGFRA (D842V), which represents 9% of all mutations with GISTs, has been shown to convey complete resistance to TKI therapy.

Techniques testing for abnormalities in chromosomes: fluorescence in situ hybridization (FISH)

Molecular abnormalities do not only occur at the level of the individual gene; it is well known that certain tumors show chromosomal alterations which are key to their development and can have clinical value. There are multiple techniques for assessing these alterations, but the most commonly used is FISH. The basic steps in a FISH procedure include the fixation of the DNA, as either metaphase chromosomes or interphase nuclei, on a slide. The DNA is then denatured in situ, so that it becomes single stranded. This target DNA is then hybridized to specific DNA probe sequences which are labelled with fluorochromes to allow for their detection. The labelled probe is added in excess so probe binding to target DNA occurs. Fluorescence microscopy then allows the visualization of the probe on the target material. Analysis of the probe signals include observation of gain of signals, loss of signals, positioning of signals or fusion of signals.

Table 20.1 Troubleshooting FISH

Problem	Possible cause	Possible solution
Slide background	Inadequate post-hybridization wash	Ensure correct wash solution and temperature. Re-wash slide.
	Inadequate cleaning of glass slides	Clean slides in ethanol and wipe dry with lint-free paper.
Weak or no signal	Specimen inadequately denatured	Ensure correct denaturation solution and temperature. Increase denaturation time.
	Specimen slides not aged	Age slides for 24 hours at ambient temperature before use.
	Probe not added	Allow probe to thaw completely before use. Vortex probe. Pipette slowly.
	Probe inadequately denatured	Ensure correct temperature of water bath.
	Counterstain is too bright	Remove coverslip. Re-wash in 2x SSC/ 0.1% NP-40 at ambient temperature. Dehydrate slides; reapply counterstain.
	Probes improperly stored	Store probes at –20°C (in dark).
Distorted chromosome morphology	Specimen over-denatured	Ensure correct denaturation solution and temperature. Repeat on new specimen with reduced time of denaturation.

Commercially available probes are usually directly labelled with the fluorochrome directly attached to the probe nucleotides. This technique involves no other detection of the probe before analysis. Probes may also be indirectly labelled by incorporation of a hapten, e.g. biotin or digoxigenin, into the DNA via nick translation. The probes are then detected using a fluorescently labelled antibody, e.g. strepavidin and anti-digoxigenin. We do not cover the complexities of designing, manufacturing and validating 'home-brew', Laboratory Design Test (LDT) ISH assays in depth within this edition for the simple reason that a significant majority of laboratories no longer utilize anything but commercially manufactured probes. However, there is widespread use of Research Use Only (RUO) probes which laboratories have themselves validated for their clinical use and CE-IVD assays have by no means replaced in-house methods in their entirety. A comprehensive overview of the considerations involved in LDT probe design, manufacture and application is given in the previous edition of the text. A brief guide to troubleshooting is given in Table 20.1.

Automation of FISH processing

As with PCR-based methods, automation of FISH is highly attractive for laboratories seeking to increase throughput and standardization. Automation can be considered in the following categories:

- ***Fully automated***: Tissue sections pass directly from the oven and to a stainer. The slide is deparaffinized, digested, prehybridized, hybridized and the signal detected all in one piece of equipment. This option tends to be expensive but can accommodate large volumes of FISH.
- ***Mainly automated***: Requires the paraffin wax to be manually removed and tissue digestion completed before placing it on the stainer.
- ***Semi-automated***: Paraffin wax removal, digestion, pre-hybridization and hybridization are performed either manually or using a hybridizer before finishing the detection part of the staining. Commercial stand-alone hybridizers perform pre-hybridization and hybridization automatically. Some do not require removal of the slides for the washes and the temperatures and times are programmed into the equipment.

Types of probes available for use in FISH

Several types of commercially available probes are available:

Dual-color/single-fusion probes

With these the DNA probe hybridization targets are located on one side of each of the two genetic breakpoints in the specific translocation, e.g. chromosomes 9 and 22 in the case of the Philadelphia chromosome associated with chronic myelogenous leukemia or acute lymphoblastic leukemia.

Extra-signal (ES) probes

These are designed to reduce the frequency of normal cells with an abnormal FISH signal pattern due to random co-localization of probe signals in the nucleus. In this type of probe set, one larger probe (labeled in one color) spans one breakpoint in the specific translocation, while the other probe (labeled in another color) flanks the break-point of the other gene involved in the translocation (Fig. 20.2).

Dual-color/break-apart probes

These are used when a specific gene may have several different chromosomal partners and are designed so that the DNA sequence on either side of the breakpoint in a specific gene is labeled in two colors. When the gene is disrupted due to a translocation, the probe is seen as two separate colors, red and green, rather than as one fused signal pattern, yellow (Figs. 20.2 and 20.3).

Dual-color/dual-fusion probes

This probe set is designed to reduce the number of normal nuclei showing an abnormal signal pattern due to random co-localization. Large probes, in different colors, span both breakpoints involved in the rearrangement. In a truly abnormal cell, two fusion signals are generally seen, representing the specific chromosomal translocation, as well as a red and a green signal representing the normal and uninvolved chromosomes (Fig. 20.2).

Uses of FISH

There are three ways in which this is helpful for clinical purposes.

1. Chromosomal alterations may contribute to diagnoses, this is particularly true in:
   - Many sarcomas (Henderson-Jackson et al., 2015), e.g. EWS translocation in the Ewing's sarcoma family and MDM2 amplification in liposarcoma.
   - Many hematolymphoid neoplasms, e.g. t (11;14) in mantle zone lymphoma and MYC amplification in Burkitt's lymphoma.
   - Some carcinomas, e.g. NUT translocation in NUT carcinoma and TFE3 in certain renal cell carcinomas.
2. Some chromosomal alterations convey useful prognostic information, e.g. co-deletion of 1p19q in glial tumors is associated with better prognosis.
3. Other chromosomal alterations are important in predicting response to treatment, e.g. HER2 amplification in breast and gastric carcinomas and ALK translocation in lung carcinomas.

Limitations of FISH

Despite its many practical uses, FISH has several significant limitations. Firstly, the ability to interpret FISH testing is heavily dependent on the quality of the tissue under investigation as reliable testing may not be possible in small, poorly representative tissue samples. Even in those samples which are high quality, there remains considerable difficultly in distinguishing between tumor and non-tumor cells, and so FISH requires highly skilled assessors and is labor intensive.

In an effort to standardize the assessment of specimens by FISH, the technique has been clinically validated through trials and strict guidelines have been established for the assessment of particular tumors. These guidelines typically mandate that certain numbers of cells be assessed and give the cut-off points for positivity, both in the case of translocations and amplification.

To ameliorate the labor-intensive nature of identifying and counting cells in FISH, particularly in the context of increasing numbers of tumors requiring testing, software based on validated and accredited algorithms has been developed for the counting of cells and assessment of probe relationships. Companion diagnostics have begun to commercialize such assays for clinical use.

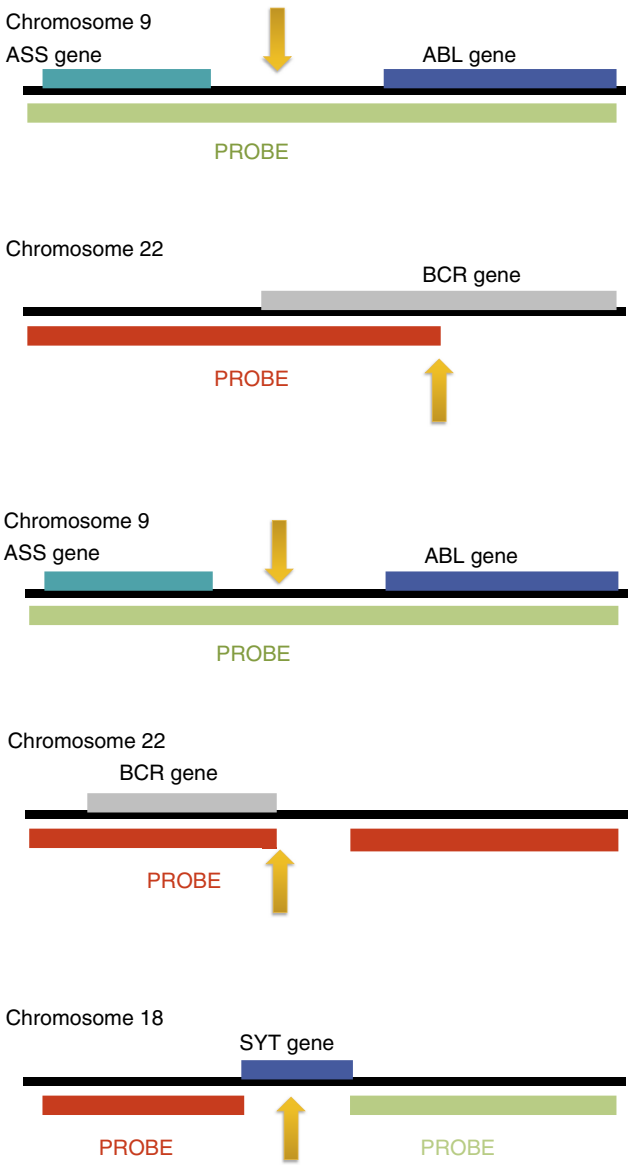
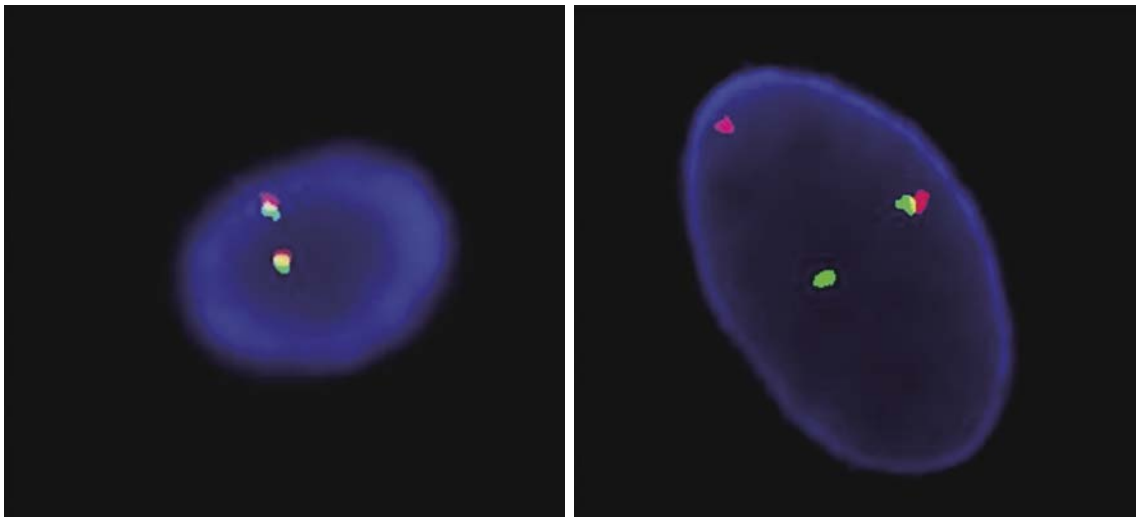


Fig. 20.2 Diagrams to show the use of dual color (red, green) probes in ISH. The breakpoints are shown as arrows. (**a**) Here there are dual-color probes showing the normal pattern of signal for chromosomes 9 and 22. (**b**) The Philadelphia chromosome mutation results from the translocation between chromosomes 9 and 22 in chronic myelogenous leukemia (CML) and some cases of acute lymphocytic leukemia (ALL), at the breakpoint shown. The extra signal is due to the presence of the ASS gene, also labeled, which remains on the derivative chromosome 9, following the translocation. The two fusion signals arise due to the fusion of part of the red signal on chromosome 9 with part of the green signal on chromosome 22, and vice versa. (**c**) Diagram illustrating the design of a dual-color, break-apart probe. This probe is used to identify translocations involving the SYT gene on chromosome 18 seen in a majority of synovial sarcomas. The probe is labeled with color markers, forming a yellow or fused signal in a normal cell. Any translocation in an abnormal cell will disrupt this fused yellow signal, creating separate red and green signals.



a b

Fig. 20.3 Representative FISH images of the SYT dual-color, break-apart probe on interphase cells from a bone marrow sample. The SYT gene is located on chromosome 18 and translocations involving this region are seen in the majority of synovial sarcoma patients. **(a)** Two yellow or fused signals (both SpectrumOrange™ and SpectrumGreen™ present) are seen, which is the normal signal pattern. **(b)** One fused yellow signal is seen, representing the normal chromosome 18, as well as a separate red and green signal, indicating that a translocation has occurred. The SYT gene is translocated to the X chromosome and forms an abnormal fusion protein with either the SSX1 or SSX2 gene. This analysis was performed in an interphase cell and, therefore, the specific translocation partner of the SYT gene cannot be determined.

FISH methodologies

FISH is generally performed using commercialized kits, and so the exact details of validation and implementation vary by manufacturer, but an overview of the essential steps and their variations is valuable. As discussed previously, whether labs are sourcing 'home-made', RUO, or CE-IVD labelled FISH probes, a variety of 'on-license' and in-house methods are applied in using these probes. Following is an overview of an in-house developed approach and a contrasting commercial method for HER2 FISH preparation and some guidelines for interpretation of HER2 and ALK.

FISH set-up

This is the basic set-up procedure for use with either commercially available or home-brew probes. The denaturation times and temperatures may need to be adjusted depending on the specific probe used, e.g. whole chromosome paint versus centromere-specific probes and the tissue type (peripheral blood specimen slide or amniocyte coverslip). Different commercially available probes are provided either pre-denatured and in solution with the appropriate hybridization buffer, or require to be prepared with the appropriate hybridization buffer and distilled water before denaturation (step 10). The two alternatives to denaturation of the slides/coverslips and probes are shown in step 12 (denaturation solution) and step 17 (all-in-one co-denaturation and hybridization system). The stringency of the post-wash solutions is important to remove non-specifically bound probes, this may be altered by changing the salt concentration, the temperature, or the time in each solution. Some specimen types require pretreatment before denaturation, removing cytoplasmic proteins of the cell membrane and allowing greater accessibility of the DNA. Pretreatment is generally performed using fresh pepsin in an acid solution. The basic FISH set-up method is the same regardless of whether the gene in question demonstrates an amplification or translocation abnormality. Often, the only difference is the probe applied to the tissue section. However, robust validation or verification in-house will provide the laboratory with information specific

to each marker and tissue type. Practically, the workflow is streamlined if all FISH probes and tissue types can adopt the same set-up protocol, however this may not always be possible and the variables discussed above may need to be addressed at the time of validation or verification.

Reagent preparation

10M NaOH

Add 200g NaOH pellets to 500 ml distilled water.

Citrate buffer

Dissolve 3.1g citric acid in 1500 ml deionized water. Mix thoroughly. Measure the pH, expected to be approximately 2.65. Adjust to pH 6.0 by using 10M NaOH. This is made up fresh for each run.

Pepsin buffer

Dissolve 9.0g NaCl in 920 ml of distilled water. Mix thoroughly and measure pH. Adjust to pH 2.8-2.9 with concentrated HCl. Bring the volume to 1000 ml with distilled water. This is made up fresh for each run.

Post-hybridization wash

Add together 50 ml 20X saline sodium citrate (SSC), 423 ml distilled water and 1.5 ml Nonidet P-40 (NP-40). Mix thoroughly. Measure the pH. If pH is less than 7.0 add 10M NaOH, if pH is above 7.5 add HCl. Ensure the final pH is between 7.0 and 7.5. Top up to 500 ml with distilled water. This solution should be changed after two uses.

Day one

1. Heat slides to melt the wax (temperature needs to be above 56°C).
2. Take slides to water.
3. Place denaturation solution in water bath at 73°C if following step 12.
4. Treat slides in freshly prepared citrate buffer at 94–99°C for at least 30 minutes. This can be achieved using a microwave or a water bath.
5. Wash slides in distilled water for 10 minutes.
6. Weigh out 0.1 g pepsin and measure 5 ml pepsin buffer. This should be sufficient for approximately 12 slides, dependent upon tissue size.
7. Remove slides from the water, shake any excess and dry the underside.
8. Position slides onto a hot plate and drop the enzyme solution onto the slide to cover the entire section. Leave for 40 minutes at 37°C.
9. Transfer slides to distilled water for 10 minutes then remove and dry.
10. Pre-warm probe to room temperature for about 5 minutes. If probe does not need to be denatured, aliquot 10 µl for each 22 x 22 mm target area, cover with a glass coverslip and seal with rubber cement. If probe needs to be denatured, aliquot 7 µl hybridization buffer, 2 µl ddH_2O, and 1 µl probe into a microcentrifuge tube. Keep probe in darkness as much as possible. Return probe to fridge / freezer as soon as possible. The exact volume of probe to be applied will be dependent upon the size of the tissue.
11. Vortex probe briefly and centrifuge for 2–3 seconds.
12. If using denaturation solution, denature the slides for exactly 2 minutes in the pre-warmed denaturant at 73°C.

Note

A maximum of three slides should be denatured at one time to maintain the correct denaturation temperature. In addition, denaturation temperatures and times may vary depending on tissue type and type of probe.

13. Dehydrate the slides in a cold ethanol series (70%, 80% and 100%) for 2 minutes each.
14. Wipe the back of the slides and place on a 37°C slide warmer to dry completely. Leave slides on slide warmer until ready to apply probe mixture.
15. If denaturing the probe, denature the aliquoted probe mix for 5 minutes in a 73°C water bath. Vortex probe briefly and centrifuge for 2–3 seconds.
16. Apply 10 µl probe mix to target area and cover with a 22 × 22 mm glass coverslip. Seal with rubber cement.
17. If not denaturing probes and slides separately, probes and slides can be co-denatured on, e.g. the ThermoBrite™ Denaturation and Hybridization system from Abbott Molecular Inc. for 3 minutes at 73°C.
18. Following either form of denaturation, incubate slides at 37°C overnight in a humidified chamber (place moist sponge or paper towels in an airtight, opaque container).

Note

Slides may be left in the ThermoBrite™ instrument for hybridization at 37°C for 4–18 hours. A minimum of 4 hours of hybridization is recommended for any probe.

Day two

1. Warm glass Coplin jar of post hybridization wash to 73 ± 1°C. Do not wash more than three slides at a time, to ensure the correct wash temperature is maintained.

2. Remove coverslip and rubber cement from hybridized slides. Keep slides covered as much as possible and away from the light.
3. Wash slides in post-hybridization wash at 73°C for 4 minutes. Agitate slides for 1–3 seconds.
4. Transfer the slides to a rack and wash in distilled water.
5. Allow the slides to dry while protected from the light.
6. Apply 2 µl of DAPI I or DAPI II to slides and cover with appropriately sized glass coverslip. The volume of DAP I will be dependent upon the size of the tissue. Seal the coverslips with clear nail varnish.
7. Keep the slides covered or in the dark to preserve the signal.

Note

a. DAP I is a carcinogen.
b. For batching FISH samples or running a FISH procedure with many steps and solution changes, automated FISH processing may be advantageous. One such pre-programmed automated system is supplied by Abbott Molecular Inc., the VP 2000™ Processor which may be used for a variety of functions, e.g. deparaffinization and pretreatment of FISH samples, histology/cytology staining, special stains of chromosomes and routine slide washing.

Specific FISH procedure: HER2 FISH (PathVysion™)

Sample requirements

Formalin fixed, paraffin wax-embedded breast cancer tissue blocks should be cut into 4 µm sections. Slides are then floated in a protein-free water bath at 40°C. Each section is mounted on the positive side of an organosilane-coated slide to minimize the loss of tissue during processing. The slides are air dried. An H&E slide, scored by a pathologist, should accompany each specimen, with the areas of tumor to be scored clearly delineated. Control slides (one negative and one positive) must be run at the same time as the patient slides in each specimen processing run to ensure accuracy of signal analysis and to monitor assay performance. If the FISH assay on the control slides does not work, then the patient analysis cannot be reported. In addition, control slides must be run with each new lot of the PathVysion™ probe kit. A more detailed reference of the procedure may be obtained from the PathVysion™ HER2 DNA Probe Kit product data sheet (Abbott Molecular Inc.).

Solutions needed for the VP 2000™ Processor

70%, 85%, 95% ethanol

Post-hybridization wash buffer
(only one wash buffer is used in this procedure)
2x SSC/0.3% NP-40

Protease I reagent
25 mg pepsin/lyophilized per 50 ml 0.01 M HCl. Make fresh.

Pretreatment reagent, 1 M sodium thiocyanate
Keep covered at ambient temperature. Expiration: 6 months.

0.2 M HCl
Keep covered at ambient temperature. Expiration: 6 months.

10% buffered formalin

PathVysion™ HER2 DNA probe kit (Abbott Molecular Inc.)
DAPI I

Day one

Heat slides overnight at 56°C (hot plate or oven).

Day two

Slide pretreatment (as run on the VP 2000™ Processor).

1. Deparaffinization in Hemo-De (non-toxic solvent similar to xylene) for 5 minutes at ambient temperature.
2. Repeat twice.
3. 95% ethanol for 1 minute at ambient temperature.
4. Repeat.
5. 0.2 M HCl for 20 minutes at ambient temperature.
6. Rinse in water for 3 minutes at ambient temperature.
7. Place in pretreatment reagent for 30 minutes at 80°C.
8. Rinse in water for 3 minutes at ambient temperature.
9. Place in protease treatment for 10 minutes at 37°C.
10. Rinse in water for 3 minutes at ambient temperature.
11. Fix in 10% buffered formalin for 10 minutes at ambient temperature.
12. Rinse in water for 3 minutes at ambient temperature.
13. Dehydrate for 1 minute each in ethanol series (70%, 85%, and 95% ethanol).
14. Air dry on drying station for 3 minutes at 25°C.
15. Proceed to FISH procedure.

Notes

a. Before each run on the VP 2000™ Processor, each basin should be filled with 470 ml of the appropriate reagent. After approximately 15 runs, all solutions should be discarded and the basins refilled with fresh reagents.
b. The fixative step 11 helps to reduce tissue loss during denaturation.

FISH procedure

See general FISH procedure described previously. It is recommended that denaturation solution (steps 10 &12) is used to denature the slides. Denature slide(s) at 72 ± 1°C for 5 minutes.

Day three

1. Wash slides in 2× SSC/0.3% NP-40 at 73 ± 1°C for 2 minutes.
2. Air dry slides in the dark.
3. Apply 20 µl DAPI I counterstain and cover with a glass coverslip.

Signal analysis

Using a 40× objective, scan several areas of tumor cells within the region corresponding to that designated by the pathologist on the H&E section. Select an area of good nuclei. Using a 100× objective, begin analysis in the upper left quadrant of the selected region and scanning from left to right, enumerate the number of signals within the nuclear boundary of each evaluable interphase cell.

Note

a. Do not score nuclei with no signals or signals of only one color.
b. Only enumerate in areas of invasive cancer (not DCIS) for breast tumors.
c. Avoid areas containing necrosis, ambiguous nuclear borders, weak signals, noisy background and insufficiently counterstained nuclei.
d. Do not count nuclei which are overlapping as not all signals will be visible. Diffuse signals can have a fuzzy or elongated DNA fiber appearance and should be recorded as a single signal.
e. More information and example images can be found in the appropriate package inserts.

Example of procedure for HER2 and ALK

1. Thirty interphase cells are analyzed by one scientist for HER2 (a minimum of twenty interphase cells must be analyzed) and fifty interphase cells are analyzed by one scientist for ALK.
2. Results are recorded as the number of signals for the HER2 probe and the number of signals for the CEP (chromosome enumeration probe) 17 probe (the centromere of chromosome 17). For ALK the results are recorded as the number of fused cells and the number of cells containing a re-arrangement.
3. The ratio of the average copy number of HER2 to CEP 17 signals is calculated.
4. If the ratio of HER2 to CEP 17 signals is borderline between 1.8 and 2.2, then a second technologist will count an additional 30 interphase cells and a new ratio is calculated.
5. Due to the temporary nature of prepared FISH slides, it is recommended that a digital image is taken of the areas of the tissue analyzed using software such as MetaSystems. If that is not available, a schematic drawing of the section of tissue analyzed should be made to indicate the area analyzed by each scientist.

Interpretation of results

Many manufacturers provide interpretation guidelines for each specific probe. It is important to familiarize yourself with these guidelines before beginning analysis of any new gene. The guidelines for HER2 and ALK are provided briefly here as examples of the two main categories of FISH testing usually performed, amplification and translocation.

HER2

These guidelines are based on the UK recommendations 2014 for breast cancer (Rakha et al., 2014).

1. Dual-probe HER2/CEP 17 ratio <2.0 **and** an average HER2 copy number <4.0 signals per cell **= negative for HER2 amplification.**
2. Dual-probe HER2/CEP 17 ratio <2.0 with an average HER2 copy number 4.0-6.0 signals/cell **or** a ratio of 1.80–1.99 **= borderline / not amplified.** In these instances, count more cells **or** retest on an alternative specimen / different tumor tissue block or resection if original specimen is a biopsy.
3. Dual-probe HER2/CEP 17 ratio ≥2.0 **or** an average HER2 copy number ≥6.0 signals per cell **= positive for HER2 amplification.**

ALK

These guidelines are taken from Vysis ALK Break Apart FISH probe kit package insert.

1. Cells are considered **negative** (non-rearranged) when:
- Red and green signals are adjacent or fused and can often appear yellow. Red and green signals which are less than two signal diameters apart are considered as a single fused signal.

- There is a single green signal without a corresponding red signal.

2. Cells are considered **positive** (re-arranged) when:
   - At least one set of red and green signals are two or more signal diameters apart.
   - There is a single orange signal without a corresponding green signal in addition to fused and/or broken apart signals.

Samples are considered:
Negative if <5 cells out of 50 are positive.
Positive if >25 cells out of 50 are positive.
Equivocal if 5–25 cells out of 50 are positive. In these instances, an additional 50 cells should be counted. The first and second count readings are added together and a percent is calculated. If the percent positive cells is <15%, the sample is considered negative. If the percent positive cells is ≥15%, the sample is considered positive.

Alternative techniques for examining chromosome structure

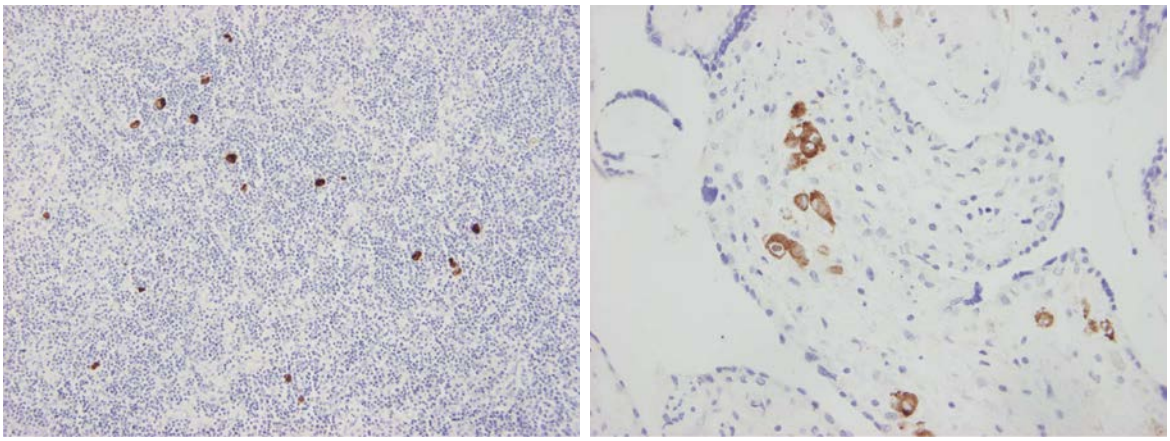
Although FISH dominates the assessment of chromosomal abnormalities, alternative testing methods using DNA or RNA assessment exist. Next-generation sequencing (NGS) is discussed below and can be used to investigate chromosomal abnormalities by assessing the DNA sequence. However, this requires high quality DNA, which is frequently not available and is subject to other substantial technical challenges, which have not been entirely resolved to the degree necessary for clinical use.

RNA assessment can, depending on the clinical situation, either be used as a surrogate or a complement to FISH assessment. The principle is that translocations generate particular RNA transcripts which can be detected using PCR and used to infer the nature of the translocations. In order that appropriate primers can be selected, it is important to know in advance the number and nature of the transcripts formed by the specific translocation under investigation. For example, ALK translocations in lung carcinomas generate at least eight different RNA transcripts and so their detection would require the use of at least sixteen specific primers. As PCR requires specific sequences to be targeted, whereas FISH assesses large areas of the chromosome, PCR is potentially less sensitive for translocation detection and this has several implications. If FISH is considered to be the gold standard for detecting translocations, PCR protocols need to be validated both clinically and technically when PCR is to be used as a surrogate for FISH in clinical practice. It is also important to appreciate that the full list of possible transcripts formed by ALK translocations is not known and as a result it is not possible to produce primers for every possible type of translocation. Using this technique will inevitably lead to the generation of false negative results which both laboratory staff and oncologists must be aware of.

In certain clinical circumstances, it may be helpful to use a combination of FISH and a PCR based technique. For example, translocations of the EWS gene (found in most cases of Ewing's sarcoma) can involve various partner genes, i.e. the gene to which EWS translocated, the precise identity of which has implications both for diagnosis and management. Ewing's FISH covers many of these partners and so may be used for the initial phase of testing, but PCR is required to assess the particular transcript generated and therefore, the partner gene.

Techniques testing for abnormalities in RNA: in situ hybridization (ISH)

This is a method which localizes and detects specific mRNA sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. It involves denaturing DNA and RNA strands using heat. A probe, a labelled complementary single strand, is incorporated with the DNA/RNA strands of interest. The strands will anneal with complementary nucleotides bonding back together with their homologous partners when cooled. Some will anneal with the original complementary strands, but some will also anneal or hybridize with the probe. As probes increase in length, they become more specific and the chance of a probe finding a homologous sequence other than the target sequence decreases as the number of nucleotides in the probe increases. A longer probe can hybridize less specifically than shorter probes. Optimal probe size for ISH is small fragments of approximately 200–300 nucleotides. However, probes as small as 20–40 bps or some as large as 1000 are in use.



a b

Fig. 20.4 Examples of in situ hybridization (ISH). (**a**) Epstein-Barr virus-encoded RNA (EBER) and (**b**) cytomegalovirus (CMV). (Courtesy of Leica Biosystems.)

Uses of ISH

In molecular diagnostic laboratories, ISH is most commonly used for the detection of Epstein Barr virus (EBV) (Fig. 20.4a) and human papilloma virus (HPV) in tumor cells. This can be important both for diagnostic and prognostic purposes. Cytomegalovirus (CMV) (Fig. 20.4b), which may cause gastrointestinal ulceration in immunosuppressed patients, can also be detected using this method.

The presence of EBV has established diagnostic and prognostic significance in lymphomas and has been in use for some time. However, it has also been shown that it has prognostic significance in other tumors, such as gastric adenocarcinomas. Recent evidence has demonstrated that EBV-associated gastric adenocarcinomas have a more favorable response to PD-L1 therapy (see below).

The presence of high risk subtypes of HPV in oropharyngeal carcinomas has been shown to be a marker of good prognosis (Lowy & Munger, 2010). It is now, therefore, recommended that these carcinomas are tested for the presence of HPV. ISH is the method of choice which also allows subtyping of HPV.

Techniques testing for abnormalities of protein expression: immunohistochemistry (IHC) (see also Chapter 19)

This technique has adopted a central importance in the morphological assessment of tumors. It assesses the cellular expression of proteins by targeting the protein using labelled antibodies. Since abnormal genes can lead to the abnormal expression of proteins, IHC can be used as an indirect method of assessing for clinically relevant molecular alterations and so, can be used to guide treatment and prognosis.

Certain IHC assays have been validated as having prognostic significance in specific tumor types. For example, the expression of the mismatch repair proteins MSH2, MSH6, MLH1 and PMS2, as detected by IHC, are associated with improved prognosis in locally advanced colorectal cancers with no nodal metastases. Although not usually considered a molecular test, Ki67 level of expression as assessed immunohistochemically can be used in a similar fashion. The degree of expression is used alongside an assessment of mitotic index to grade neuroendocrine tumors and thus guides their management.

However, it is not only assessment of immunohistochemical expression in tumor cells which is clinically significant. There is growing evidence that expression of certain markers by non-tumor cells can have prognostic implications. For example, Immunoscore, assessing the level of expression of CD3 and CD8 within the stroma, has been validated as an independent marker of good prognosis in colorectal cancers and is also used in other

clinical scenarios. In addition, the emergence of immunotherapy has necessitated the validation of clinically relevant predictive markers which include, amongst others, the level of expression of proteins in lymphocytes. In current clinical practice, levels of PD-L1 expression within tumor cells is the predictive marker of choice for PD-L1 therapy in lung carcinomas. In all these cases, the clinically important cut-off for expression of these markers varies depending on the drug to be prescribed and, in some cases, on the stage of the tumor, e.g. the use of different cut-offs for first and second line therapy. As understanding of the tumor microenvironment has increased in recent years, mounting evidence has emphasized the central role of intratumoral immune cells in tumor progression. It is therefore likely that molecular assessment of the immune system alongside tumor cells themselves will be a major theme in targeted cancer therapies in the future, mandating that molecular pathology services continue to use multiple platforms, including IHC assessment.

Techniques testing for surrogate markers of molecular alterations

Although it is possible in principle, to detect all molecular abnormalities by directly examining genes, chromosomes or protein expression, in certain circumstances it may be preferable to assess an alternative surrogate marker. Two examples are the assessment for HER2 amplification in breast and gastric cancers and ALK translocation in lung carcinomas. Both can directly be assessed using FISH, but this requires highly trained assessors, high quality tissue samples and is labor-intensive. As a result, IHC tests have been developed as a screening test for HER2 amplification and as a surrogate for ALK translocation assessment.

HER2 in breast cancer

The presence of HER2 amplification in both breast and gastric carcinomas predicts good response to trastuzumab (Herceptin) therapy. The currently used model for HER2 assessment is a two-step process. This initially involves an IHC assay for HER2 protein expression by tumor cells. This works on the assumptions that:

- If there is no HER2 protein expression by tumor cells on IHC then there cannot be amplification of the HER2 gene.
- Strong membranous HER2 protein expression on IHC implies amplification of HER2.

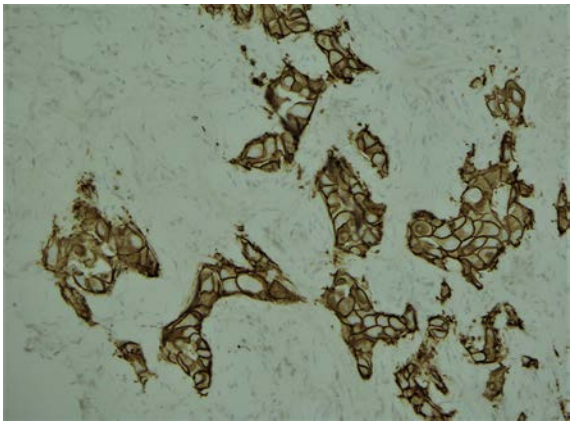


Fig. 20.5 Ductal adenocarcinoma of the breast, tested for HER2 status using the Ventana anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody. It shows strong, complete membranous HER2 expression in almost all cells, and so is regarded as positive.

Immunohistochemistry, therefore can be used to assess most tumors and FISH is required only for the limited number of tumors which show borderline HER2 expression on IHC. An approved assay for protein expression was originally launched by Dako (HercepTest) and a few years later, a validated antibody was launched by Ventana (4B5 clone). Both tests are in use today and their implementation, of course, required clinical studies to first demonstrate that the IHC assay was as robust a predictive marker as FISH testing (Fig. 20.5).

ALK in non-small cell lung cancer (NSCLC)

Crizotinib (Pfizer) has been shown to be beneficial in approximately 3% of NSCLC which bear ALK translocations and it is licensed for treatment in these cases. The companion diagnostic used during the trials originally demonstrating its efficacy was Vysis FISH probes from Abbott. As NSCLC typically presents at an

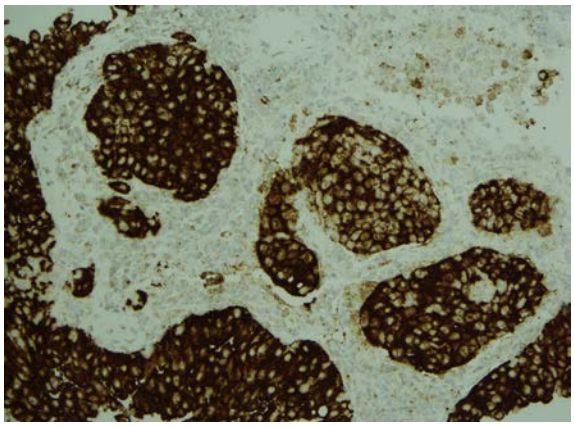


Fig. 20.6 Lung adenocarcinoma, tested for ALK status using the Ventana D5F3 Rabbit Monoclonal Antibody. It shows strong granular cytoplasmic staining, and so is regarded as positive.

advanced stage and survival is typically short, it is vital that diagnosis is completed as rapidly as possible. It is therefore easy to see why FISH, being time consuming and labor-intensive, was far from ideal in the assessment for ALK translocations. Immunohistochemistry has therefore become the favored surrogate for FISH testing in most European laboratories. The anti-ALK D5F3 antibody commercialized by Ventana is now FDA approved for first-line testing in NSCLC prior to targeted therapy prescription (Fig. 20.6).

Immunohistochemistry is not the only technique which can be used as a surrogate for ALK FISH. The use of RNA in inferring chromosomal alterations has already been discussed above. Additionally, ALK translocations can be assessed from extracted DNA using deep sequencing technology. This technique (discussed below) would, in theory, not miss any alterations but requires strong clinical validation, bioinformatics and a larger amount of DNA than the others described above.

Future directions

Companion diagnostics have also developed antibodies which specifically bind mutated proteins which can be used as a surrogate or to complement other techniques in some clinical circumstances. For example, there is an antibody which specifically binds the protein product of V600E-mutated BRAF but not that of the wild-type BRAF. This assay can, therefore, be used in place of PCR-based techniques.

Techniques testing for multiple molecular alterations: multiplex testing

With the growing number of molecular markers proven to have clinical relevance for various tumors, it is increasingly common to need to perform tests for multiple molecular targets on a single tissue sample. This is known as multiplex testing.

Next-generation sequencing (NGS): single platform multiplex testing

The most obvious example of multiplex testing is the use of a single platform which tests for several mutations, e.g. actionable panels used in NGS platforms. To fully address NGS methods, in view of their complexity, multiplicity and relatively novel nature, would require considerable exposition. Without doubt they will require bespoke forms of pre-analytical processing, sample preparation, DNA extraction, analysis and interpretation. Whilst many laboratories may be able to modify existing processes, the DNA extraction needed to meet the demands of NGS and other aspects such as the use of highly demanding bioinformatics to interpret data are entirely unprecedented and make it difficult. NGS pipelines as a whole can be thought of as requiring the following essential components.

Library preparation

Fragmentation and/or PCR amplification of DNA is used to select strands of a specific length. Double-stranded copies of the region of interest are then produced and oligonucleotide adapters are 'tagged' on-to the end of fragments intended for sequencing. The products of this stage are referred to as the 'library'. Quantification is then performed, usually using a fluorescence based method, so that the DNA entered into the next stage is precisely measured, ensuring economical and unbiased sequencing.

Sequencing

The various NGS platforms on the 'market' employ a variety of different approaches. Examples include, but are not limited to, classical PCR amplification,

variants of pyrosequencing and biochemical methods dependent upon ion semi-conduction. Individual strands of DNA present within the library are analyzed, producing a multitude of unique 'reads', each providing Sanger sequencing-like information which is quantitative in nature, quite unlike classical sequencing.

Bioinformatic interpretation

Traditional Sanger sequencing or pyrosequencing data are interpreted manually, or more commonly using qualitative software, by suitably trained interpreters. The huge number of individual 'reads' (the sequence of each individual strand analyzed, often hundreds or thousands of each amplicon in NGS analyses of solid tumors) in the output of NGS makes immediate analysis by scientists impossible. The many gigabytes of data produced must instead be analyzed using tailored bioinformatic software which aligns the multitude of sequence reads against known reference sequences. They must quantify the proportion of any variants present at particular nucleotide positions and, where possible, eliminate apparent variants which derive from sequencing errors or DNA defects not due to genuine mutations. Bioinformatic interpretation of data is essential to NGS pipeline implementation.

Multi-platform multiplex testing

Although multiplex testing is often assumed to involve the testing of multiple mutations using a single platform, there is no reason why this should necessarily be the case. Indeed, an integrated molecular pathology service assessing single specimens for a range of molecular markers using multiple platforms in parallel, is undertaking the same task, and may be the most effective means of doing so. This is, therefore, an alternative means of conceptualizing 'multiplex testing'.

The advantage of multiple platforms is that they confer the flexibility to assess potentially any clinically relevant target, regardless of the type of testing modality required. Such a system is particularly suited to testing for the actionable targets currently required in NSCLCs where EGFR1 mutations are detected using PCR, ALK translocation for IHC or FISH and PD-L1 expression by IHC. A further example is colorectal cancer, in which KRAS, NRAS and BRAF could be assessed separately, on multiple platforms or groups on an NGS or real-time PCR platform, but assessment of mismatch repair protein expression would nonetheless require IHC assessment.

New avenues in molecular techniques: circulating tumor DNA (ctDNA)

The ability to assess for specific mutations using plasma rather than tissue is a promising technology which has now become reality. In healthy individuals, plasma contains DNA released during lysis or necrosis from many cells around the body. It is known that DNA shed from tumor cells can also be detected in the plasma of individuals with cancer. It is an appealing thesis that this DNA could be collected from a simple blood sample in lieu of invasive tumor sampling.

This approach is, however, not without its own difficulties. Tumor DNA in blood is highly fragmented and is scanty. It is important that DNA extracted from plasma avoids contamination by white blood cells and so it is essential that important measures be taken against lysis of cells within the sample. If classical EDTA tubes are used for collection the plasma must be isolated as soon as possible after blood sampling. Alternatively, specially designed tubes which resist lysis can be used and several are currently available. Techniques for DNA extraction should be well-established, either manually or on automated equipment, again avoiding contamination by blood cells.

Testing should use highly sensitive validated techniques. Although real-time PCR is currently the technique of choice and various types are available, many interesting and promising research projects involving NGS are underway. Companion diagnostics are also working on CE-marked/IVD assays for specific genes.

Qiagen and Roche Molecular Diagnostics have both launched plasma based validated assays to assess EGFR mutations in lung cancer patients and this has now been implemented in certain diagnostic laboratories.

Although the potential of this new technique cannot be overstated, it is important to bear in mind that it is by no means perfect. All studies to date and the information supplied with the commercialized kits, state that sensitivity of testing is, on average, below 70% in patients with metastatic lung cancer. When compared to the almost 98% sensitivity seen in FFPE specimens, the difference is stark. Consequently, it is essential that all reports based on these techniques include the caveat that a negative result does not necessarily exclude the presence of the mutation, and this seriously hampers the clinical utility of ctDNA. However, this does detract from the fact that plasma mutation testing represents an extremely useful adjunct to tissue testing. In particular, it may prove to have unparalleled use in the monitoring of lung cancer patients who are taking TKI therapy.

The clinical validation of real-time PCR assays for EGFR and other genes, and ultimately for NGS panels, will need to take into account variability in the amount of circulating free DNA in each type of tumor, depending on the clinical stage. The difficulty will be how to deal with a negative result, in light of the relatively high rate of false negative results.

The challenge: choosing the most appropriate molecular technique

In routine practice the choice of technique for assessment of a specific alteration is always a compromise. In Europe, laboratories have the liberty to choose whichever technique is deemed most suitable, but it is different to the FDA requirements in the United States. Choice should take into account the nature of the specimens expected, the turnaround time needed for effective clinical management, the platforms which are already in place in the laboratory with which the scientists or technicians are familiar, and the cost. Tests should be accredited, and here laboratories must make a decision. They may select either a CE-marked IVD assay, which will make the accreditation procedure easier, or, they may decide to validate a home-brew test, which will require more work for the final validation according to ISO criteria (Long-Mira et al., 2015).

Ideally a backup technology should be validated for each test to make sure that there is no service interruption in case of technical problems. Backup techniques could also be an agreement with another laboratory, allowing samples to be redirected until the problem is solved.

The technical challenges of molecular testing

Although many challenges abound in all aspects of the specific molecular testing modalities discussed previously, the two serious technical challenges which apply to all techniques are small amounts of substrate for testing and the deleterious effects of formalin fixation.

Tissue size and DNA available for testing

It is worth stressing, although self-evident, the fact that ultimately molecular testing can only be performed using the tissue available and this is the excess remaining once morphological assessment has been completed. Given that targeted therapies, and by extension molecular techniques, often contribute most to those patients who are so unwell or so frail that they cannot undergo aggressive surgical management, the specimens usually available for molecular assessment are small biopsies. Often, therefore, molecular pathology services receive limited amounts of tissue with which multiple molecular assays must be performed. In part, this difficulty has been circumvented in recent years by adaptations in the pathologists' practice, ensuring that tissue remains for molecular assessment. It is now common for histopathology departments to produce paraffin blocks or clots from cytological specimens so that standard histological procedures can be performed on them, and in this way, molecular testing can also be performed on both cytological and histological material.

The problem is not solely limited to specimen size. It must be borne in mind that a given tumor sample contains not only tumor cells, but also large numbers of non-tumor environmental cells, e.g. stromal and/or inflammatory cells. This has two implications. Firstly, only a portion of the small amounts of

tissue available for testing will actually comprise the cells of interest. Secondly, in the process of assessing tumor cells for gene mutations, DNA from non-tumor bystander cells is also amplified, with the effect of diluting the target of interest at the time of DNA amplification.

The deleterious effects of formalin fixation

Given that so little useful material is available in samples used for molecular assessment, it may be assumed that what remains must be of good quality. This could not, however, be further from the truth. Counterintuitively, whilst formalin fixation is essential for accurate assessment of tumor morphology, the process degrades both DNA and cellular proteins making molecular analysis yet more challenging. In response, it is attractive to propose that formalin should be replaced by an alternative fixative. However, it is important to appreciate that the morphological assessment and classification of tumors, upon which diagnosis and management ultimately depends, relies entirely on the artifactual appearances generated by the use of formalin fixation.

Replacing formalin with an alternative fixative would require a mammoth recalibration of diagnostic criteria to the appearances of tissue generated by a new fixative across all of histopathology. Immunohistochemical techniques, which have revolutionized tumor assessment, have also been developed entirely for use in formalin fixed tissues. In routine practice, companion diagnostics offer techniques which are validated for use in formalin fixed tissue, however imperfect it may be.

An alternative approach would be to freeze all tissue at receipt. Although this improves preservation of DNA and proteins, it prioritizes DNA quality over pragmatism and accurate morphological assessment. Freezing all tumor tissue on receipt would require substantial resources and perhaps, more importantly, would seriously hamper the morphological assessment of tumors which still contributes the most to diagnosis, treatment response prediction and prognosis. Freezing of tissue may appropriately be considered an adjunct in specific circumstances, but cannot realistically replace formalin fixation.

The use of circulating tumor DNA in plasma is a similar attractive prospect as it avoids the use of formalin. However, the limitations of this approach are twofold. Firstly, morphological diagnoses cannot be made on plasma samples and tissue is still required for tumor typing, which is of paramount importance. Secondly, as already discussed above, plasma testing naturally gives a relatively high rate of false negative results. The issue of sampling is therefore key in satisfying clinical requirements and makes plasma testing extremely useful as a supplement to, rather than a replacement for, tissue testing.

Sensitivity of molecular tests

No test, in any field of medicine, is completely sensitive and completely specific. The difficulty is finding an acceptable trade-off between false negative and false positive results. Sensitivity, in particular, is a substantial problem in molecular testing because a negative result can be interpreted either as the lack of a mutation in the tumor, or as a lack of tumor in the specimen tested. Different molecular techniques have different sensitivities and this must be taken into account, together with the tumor burden in the sample, prior to testing. If, for example, few tumor cells are present in a sample, the lack of a detectable mutation may simply reflect the fact that the amount of mutated DNA fell below the threshold for sensitivity of the assay. This uncertainty would need to be communicated to clinicians. Whether a sample has sufficient tumor burden or not, is partly determined by the proposed assay, e.g. Sanger sequencing has a relatively low sensitivity for mutation detection compared to real-time PCR. Conversely, a sample containing sufficient tumor burden to deliver a valid result using real-time PCR would not necessarily be adequate for Sanger sequencing.

Whilst at present, assessments of tumor burden are most commonly carried out by trained practitioners, improved standardization of the process is possible using digital imaging systems which have been validated for particular assays. These digital assessment systems are likely to adopt even greater importance with the increased use of molecular techniques assessing broader ranges of alterations,

e.g. multiplex modalities (described above), where knowledge of tumor burden at interpretation is of the utmost importance in distinguishing relevant genetic alterations from random background alterations. Companion diagnostics are developing software to be applied to digitized H&E-stained slides, which may well become the gold standard for accredited tumor assessment prior to multiplex gene testing.

Clinical correlation in molecular pathology

The ultimate goal of a molecular pathology service is to provide clinically useful information for a patient's diagnosis, management and prognosis. It is the responsibility of molecular pathology departments to be able to provide the full range of accredited tests to clinicians in a clear and integrated fashion, within a few working days, using poor quality tissue and in a cost-effective manner. This is, to say at the least, no mean feat.

Molecular pathology reports

A molecular pathology report should be much more than a simple list of molecular abnormalities identified, and:

1. Ideally it should be provided as a supplement to the histopathology report, so that morphological, immunohistochemical and molecular features of the tumor can be combined into a complete account of the pathology.
2. It is important that the technique used is explained in the report, together with a list of the molecular alterations to which it is sensitive and the sensitivity of the technique. This becomes important if molecular testing is later performed elsewhere which generates a different result. The fact that the second test was not sensitive to the mutation detected by the first would avoid confusion. A mention of the burden of tumor included in the sample tested is also helpful. If no mutation is detected, low tumor burden would raise the possibility of a false negative result, and may prompt re-testing if clinically appropriate.
3. The report, where possible should include some interpretation of the clinical significance of the results. For example, many different mutations have been identified in the EGFR gene, a proportion of these are known to convey sensitivity to TKI therapy in lung cancers, some convey resistance and others currently have unknown implications for TKI therapy. This should be included in the report to help guide the clinical decision making.

The relevance of a mutation

The number of molecular alterations which are known to have clinical relevance remains limited. The process of establishing whether a mutation has clinical relevance is by no means simple, and involves a lengthy process of validating the value of the mutation of the marker in clinical trials. Ultimately, successful validation requires that a specific marker be shown to convey a degree of statistically significant advantage to the patient and for most markers this is only in the region of 60-70% of cases.

Identification of the clinical value for a specific mutation does not necessarily mean that the clinical value applies to all types of tumors. It has been clearly demonstrated that not all tumors with a particular molecular alteration will respond to a targeted treatment. There are other factors, mostly unknown, which complicate the relationship between molecular markers and responses to treatment. This has been best exemplified in the recent past by the example of BRAF. Anti-BRAF drugs are known to be effective in the management of BRAF-mutated melanoma, but are ineffective in the management of BRAF-mutated colorectal and lung cancers. Clearly there is limited understanding about the differing molecular biology of tumors.

Another issue is posed by the genetic instability of tumors. Tumor cells bear large numbers of mutations, but only a small proportion of these mutations actually drive cancer development, the so-called *driver mutations*. The remaining mutations, which may be numerous, are essentially incidental and result purely from the fact that these cells are prone to accumulating genetic damage, these are *passenger*

mutations. As the sensitivity of molecular techniques increases, particularly deep-sequencing, increasing numbers of passenger mutations are being detected. The result is, huge lists of mutations are returned and from this it is difficult to distinguish those mutations which can be helpfully targeted from those which are incidental.

In public health systems, therefore, there is little to be gained clinically from an exhaustive search for all mutations present in a given tumor. Instead, the selection of appropriate tests requires knowledge of which mutations are clinically relevant for a particular tumor. Not only is this preferable in terms of cost effectiveness, but it also avoids potential confusion when enormous lists of clinically irrelevant mutations are presented to clinicians.

Molecular pathology in specific tumors

Non-small cell lung cancer (NSCLC)

At present, NSCLC is routinely tested for three different molecular alterations (Cheng et al., 2012; Sholl, 2016).

EGFR mutations

TKI therapy is currently licensed and approved for the first-line treatment of advanced NSCLC and, in certain circumstances, also for second-line treatment. Prior to prescription, it is mandatory that the patient's tumor be tested for the presence of an EGFR mutation which is found in approximately 10% of Caucasian patients but over 50% of East Asian patients. These results are important, as approximately 70% of patients with an EGFR1 mutated NSCLC show favorable responses to TKI therapy, but it has also been demonstrated that patients who do not have an EGFR mutation actually have worse overall survival than with standard treatment if given TKIs. It is therefore vitally important that TKI therapy be given only to those patients with the appropriate mutation (Tan et al., 2016).

TKIs preferentially target cells which bear mutated, constitutively activated EGFR receptors but tumors which initially respond to treatment inevitably develop resistance after several months to several years. Although the molecular alterations for secondary TKI resistance are poorly understood, and are likely numerous, in approximately 50% of cases it has been shown to be associated with the selection of a population of tumor cells bearing a secondary T790M mutation within EGFR1.

Following this finding, osimertinib (Tagrisso®, Astra Zeneca) has recently been licensed and approved as second-line therapy in patients who progress under first-line TKI therapy with a secondary T790M mutation. Prior to prescribing this drug, it is mandatory that the presence of a T790M mutation be proven. This can be achieved with a repeat biopsy of the tumor, but it may represent a possible application for plasma testing.

Further research into secondary TKI resistance is beginning to identify a whole host of other molecular alterations which may underlie progression on therapy. These include HER2 and MET amplification. It is possible that these will become targets for therapy in the future.

Furthermore, there are some data to show that tumors with a TKI-sensitive mutation and a T790M mutation which advance under osimertinib treatment could benefit from a drug targeting a possible third mutation within EGFR1 at codon 797 (C797S) and candidate drugs are currently being assessed in trials.

ALK translocations

The significance of ALK translocations in NSCLC has been discussed above. Crizotinib (Pfizer) has been licensed in NSCLC showing ALK translocations. Assessment for these translocations is mandatory prior to prescription of first- or second-line therapy.

Ceritinib (Zykadia®, Novartis) is another drug targeting ALK translocated tumors which is currently licensed for ALK-translocated NSCLC which progress under crizotinib.

ALK mutations, MET mutations and several others, represent potential causes for resistance to ALK therapy and may adopt greater significance in the future.

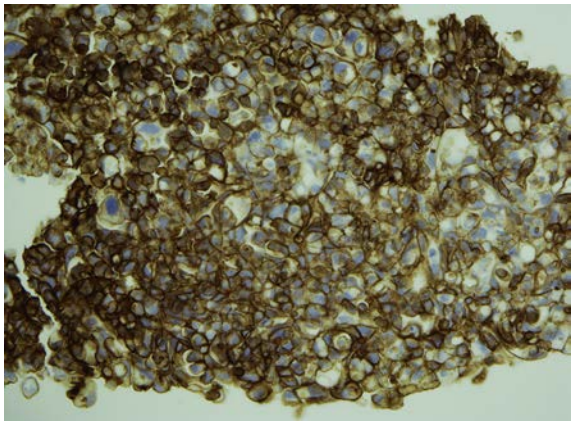


Fig. 20.7 A cerebral deposit of metastatic non-small cell lung cancer, tested for PD-L1 status using the Dako PD-L1 IHC 22C3 pharmDx test. It shows membranous expression of PD-L1 in virtually all cells, and so is regarded as strongly positive.

It is worth noting that crizotinib also targets ROS1- and RET-translocated NSCLC. Licensing has not yet been given to crizotinib for these targets, but laboratories are currently in the process of validating assays sensitive to these alterations in preparation for likely future extension of licensing.

PD-L1 expression

Recent studies have provided extremely promising data relating to the use of anti-PD-L1/PD-L1 therapy in NSCLC and in a whole host of other tumors (Hui et al., 2016). Trial data from Keynote-024 (Reck et al., 2016) have demonstrated that pembrolizumab (Keytruda®, Merck) is associated with improved progression-free and overall survival compared to chemotherapy when used as a first-line treatment in patients with advanced NSCLC showing PD-L1 expression in tumor cells. The clinical utility of PD-L1 expression assessment in this setting has been validated based on the PD-L1 IHC 22C3 pharmDx test from Dako. Other drugs targeting PD-L1 using their own IHC assays are available from other manufacturers and so it is essential that laboratories use the specific assay matched to the specific drug to be prescribed. Given the plethora of PD-1/PD-L1 targeting drugs and their associated assays, studies are currently attempting to standardize the assessment of PD-L1 expression (Fig. 20.7).

Colorectal cancer

At present, colorectal cancers are assessed for RAS (KRAS and NRAS) mutations and increasingly for BRAF mutations and mismatch repair (MMR) protein expression (Carethers & Jung, 2015; Gong et al., 2016; Nowak & Hornick, 2016). Improving knowledge of this common cancer at the molecular level has led to the development of a number of molecular classifications of this disease on the basis of the molecular alterations (Guinney et al., 2015); under one such classification, colorectal cancers are divided into:

- RAS-mutated tumors
- BRAF-mutated tumors
- Microsatellite-unstable tumors (MSI-H)
- Hypermethylated tumors.

This may be adopted in the near future due to its clinical relevance for the management of patients.

RAS mutations

A proportion of patients with advanced colorectal cancer show good responses to anti-EGFR1 therapy with cetuximab (Erbitux®, Merck Serono) or panitumumab (Vectibix®, Amgen). However, it is known that 40-45% of tumors bear a mutation within exons 2, 3 or 4 (codons 12, 13, 59, 61, 117 and 146) in the KRAS or NRAS genes and these predict no response to anti-EGFR1 therapy. Testing for these alterations is therefore mandatory prior to starting anti-EGFR1 treatment.

BRAF mutations

Approximately 6-8% of colorectal cancers carry a BRAF mutation. Interpretation of the presence of these mutations is not quite as straightforward as those alterations already discussed but generally has two possible implications:

1. The presence of a BRAF mutation *in the absence of an alteration in the MMR system* is a marker for poor prognosis.
2. The presence of a BRAF mutation *in the presence of MLH1 loss of expression* indicates that the MMR alteration is sporadic rather than familial. In essence, this excludes the presence of Lynch syndrome, an inherited disease in which sufferers are prone to developing multiple malignancies.

There is a suggestion that the presence of a BRAF mutation may have predictive value for anti-EGFR1 therapy, but this remains controversial.

Mismatch repair protein (MMR) expression

The DNA MMR system identifies and repairs errors induced in DNA during the course of DNA replication. It comprises a complex of four proteins, MSH2, MSH6, MLH1 and PMS2 which are paired into MSH2-MSH6 and MLH1-PMS2. Defects in this system increase the likelihood of sustaining DNA damage and, in particular, predispose to the development of *microsatellites*, a state known as microsatellite instability.

Approximately 15% of colorectal cancers have high levels of microsatellite instability, MSI-H tumors. Although the majority result from sporadic defects in the MMR system, a minority of cases are linked to Lynch syndrome (see above).

Testing for failure of the MMR system can be carried out in two ways:

1. IHC can be performed for each of the MMR proteins. Loss of the nuclear expression of the protein by the tumor cells is indicative of an MMR defect.
2. PCR can be used to assess microsatellite instability directly.

Concordance between these two techniques is almost 98%. Although either technique can be used, in practice, the ease of IHC assessment usually makes it the favored option. Under these circumstances, Lynch syndrome can be diagnosed in the context of:

- Loss of MSH2 expression.
- Loss of MSH6 expression.
- Loss of PMS2 expression with preserved MLH1 expression.

The loss of MLH1 expression *can* be seen in Lynch syndrome, but it can also be seen in sporadic cases. Therefore, the loss of MLH1 expression should be followed by testing for BRAF testing and for MLH1 gene promoter methylation. If either a BRAF mutation or MLH1 gene promoter hypermethylation is present, the tumor is regarded as sporadic. If neither BRAF is mutated nor MLH1 methylated, the tumor is regarded as an expression of Lynch syndrome, with the associated screening and counselling implications (Fig. 20.8).

MMR status testing has multiple uses. It is used to screen for Lynch syndrome, has negative predictive value for 5-fluorouracil therapy, has positive predictive value for immunotherapy (see page 426), and has prognostic value in pT3-4 pN0 colorectal cancers; MMR-deficient patients have an improved prognosis and so do not benefit from adjuvant chemotherapy.

Immunoscore

The scoring of CD3 and CD8-positive T-cells within colorectal cancer has been shown to be a strong and independent marker of a good prognosis. A grading system by Galon et al. (2012) has been validated and a software based algorithm (HalioDX, see Useful websites) has been developed which assesses the numbers of T-cells on digitized IHC sections.

The same system may be of predictive value in other cancers, and is being assessed currently in melanoma.

Melanoma

BRAF mutations

35–40% of melanomas bear a mutation within codon 600 of the BRAF gene. More than 80% of these mutations are the V600E type and approximately 15% are the V600K type. A range of other, rare mutations has been described.

The presence of a BRAF mutation in melanoma is a predictor of good response to anti-BRAF therapy (Shelledy & Roman, 2015; Zhang, 2015). Two drugs are currently licensed for use in melanoma, vemurafenib (Zelboraf®) from Roche and dabrafenib (Tafinlar®) from GlaxoSmithKline.

KIT mutations

Some non-sun exposed melanomas carry mutations in exons 11, 13 and 17 of the KIT gene. Even though imatinib, a TKI, is not currently licensed for use in melanoma in most areas of the UK, it is common for BRAF wild-type melanomas to be tested for KIT status because it is known that some KIT-mutated tumors respond to this drug (Kunz, 2015).

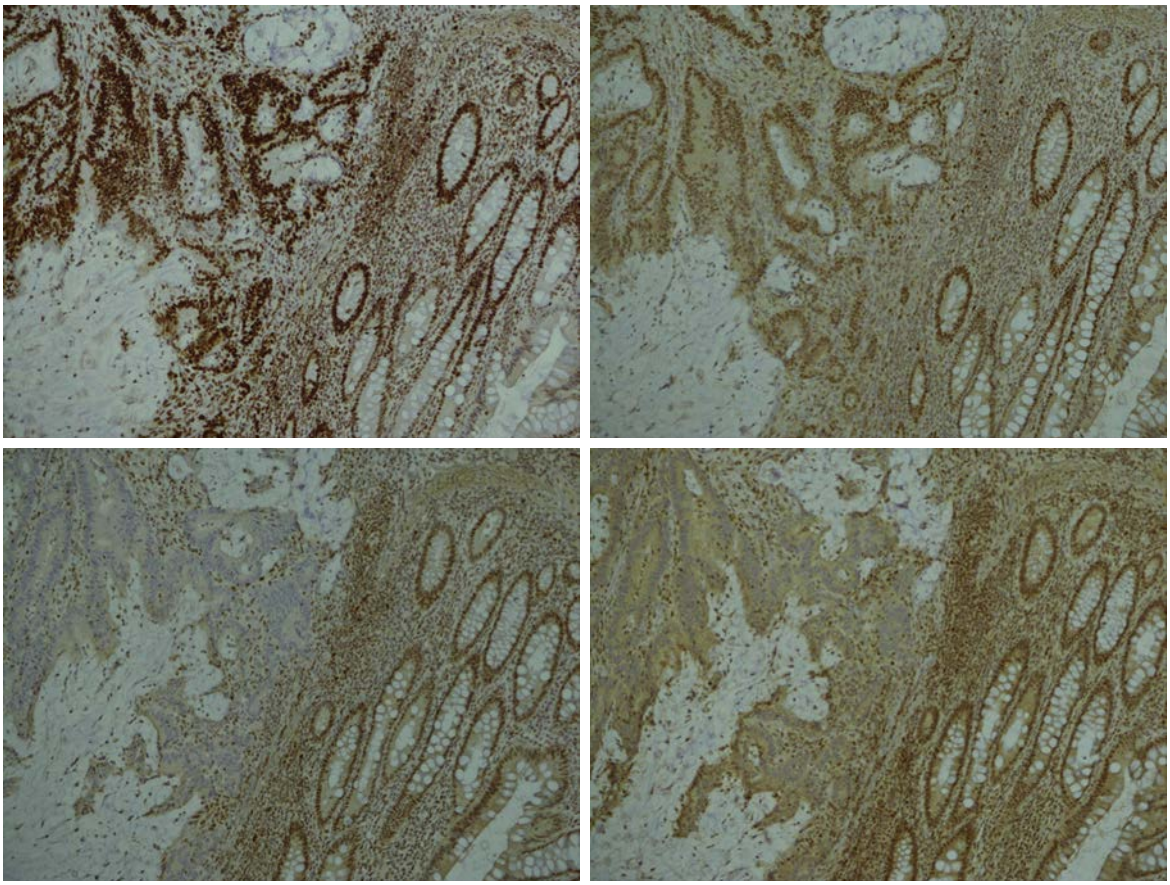


Fig. 20.8 Mismatch repair protein immunohistochemistry performed on a colonic adenocarcinoma (upper left of each image), with background colon (lower right of each image). The upper panels show preservation of nuclear expression of MSH6 and MSH2 in the tumor. The lower panels, however, show loss of expression of PMS2 and MLH1 in the tumor cell nuclei; note that expression is preserved in the nuclei of the non-tumor cells. This tumor was subsequently found to carry a mutation in codon 600 of the BRAF gene, suggesting that the tumor was not part of Lynch syndrome.

Immunotherapy

Good responses have been reported to immunotherapy regimens in melanoma (Margolin, 2016). An anti-CTLA-4 agent (ipilimumab) and a combination of anti-CTLA-4 and anti-PD-L1 therapy (ipilimumab and nivolumab) have recently been licensed. Evidence is still accruing in this area so the situation is likely to change in the near future, but at present there is no validated predictive marker for anti-CTLA-4 therapy. Currently, PD-L1 assessment is not mandatory prior to treatment with nivolumab.

Breast cancer

HER2 amplification

As previously discussed, HER2 amplification is a predictive marker of response to trastuzumab (Herceptin®) therapy and is also a marker of poor prognosis (Yersal & Barutca, 2014). HER2 status can be measured directly by FISH, or indirectly, by measuring expression of HER2 by IHC. In practice, it is common to use a two-step protocol. Immunohistochemistry is used initially, and the expression of HER2 is scored 0-1 (negative), 2 (borderline) or 3 (positive), based on the intensity and

pattern of membranous staining on the tumor cells. Negative tumors will not receive trastuzumab therapy but positive tumors will. Borderline tumors are tested with FISH, which remains the gold standard for HER2 assessment.

Oncotype DX®

There are no other routine predictive markers used in clinical practice for breast cancer (Harris et al., 2016). The Oncotype DX® Test is now National Institute for Health and Care Excellence (NICE) approved in England, and tests for a series of 21 genetic alterations (Ki67, STK15, Survivin, Cyclin D1, MYBL2, Stromelysin 3, Cathepsin L2, GRB7, HER2, ER, PR, Bcl2, SCUBE2, GSTM1, BAG1, CD68, and a series of reference genes) are used to determine prognosis in breast cancer (Cronin et al., 2007).

Gastric and esophageal cancers

HER2 amplification

In both gastric and esophageal cancers HER2 amplification predicts a good response to trastuzumab therapy but, unlike in breast cancers, it is not a marker of poor prognosis in gastric cancers. A two-step protocol can be used for HER2 assessment in the stomach and esophagus, as in breast cancers, but the criteria for grading are different (Kelly & Janjigian, 2016).

Immunotherapy

A proportion of gastric cancers are driven by either EBV infection or by MMR defects; both may predict response to immunotherapy (see below).

Molecular classification of gastric cancers

There is evidence that subtyping of gastric cancers according to the aforementioned molecular alterations may have clinical relevance, as demonstrated in data collected through the Cancer Genome Atlas (TCGA) project (Jácome et al., 2016).

Brain tumors

The recently published 2016 World Health Organisation Classification of Tumors of the Central Nervous System incorporates molecular parameters into the pathological diagnosis of many types of tumors (Louis et al., 2016). As a consequence, any newly diagnosed primary brain tumor requires assessment of IDH mutations, ATRX expression, 1p18 co-deletion and sometimes BRAF mutations, EGFR alterations, and MGMT methylation which predicts response to alkylating agents (Wang et al., 2015).

Molecular pathology across tumor types

Hitherto, the paradigm in molecular pathology has been that specific molecular markers have specific implications in particular tumor types; however, with the advent of immunotherapy, it has been found that certain markers have similar significance across multiple tumor types. As an example, the FDA has recently approved mismatch repair deficiency as an agnostic predictive marker for pembrolizumab in any type of solid tumor. Mismatch repair deficiency is one means of generating large numbers of mutations in a tumor (high tumor mutation burden, TMB), and this may in itself predict immunotherapy response. The challenge is to establish and validate clinically relevant cut-offs for each tumor type, and to develop techniques which allow practical measurement of TMB in routine diagnostic settings.

Predictive markers for non-targeted therapy

When confronted with the wide array of targets for molecular therapy, it is easy to forget that only a minority of cancer patients will ever be eligible for these treatments. The vast majority of patients with advanced, malignant, solid tumors are treated with non-targeted chemotherapy. It is well known that two patients with the same tumor will not necessarily respond in the same way to the same chemotherapy regimen. It is likely that molecular differences between tumors underlie these differential responses. Indeed, given that the mechanisms of action of most chemotherapy agents are related either to enzymes involved in DNA repair, e.g. ERCC1 and platinum-based chemotherapy,

or to the transport of molecules across the cell membrane, it seems reasonable that specific markers predicting chemotherapy response could be discovered.

However, at present, there are virtually no validated tests predicting response to non-targeted therapy, with the notable exception of MGMT methylation, which predicts response to alkylating agents in glial tumors (discussed above). The main difficulty preventing discoveries in this field is that testing would require quantification of expression of a particular gene or protein by the tumor. Though this may seem straightforward, the fact that many cells within a tumor are bystander inflammatory cells, the difficulty of quantification immediately becomes clear. Validation of robust IHC assays based on levels of protein expression would be extremely helpful. It is possible that this may become feasible in the future with the development of validated and accredited software applied to digitized slides reducing the labor-intensive nature of the work and standardizing assessment.

Perspective

Molecular pathology has the power to transform the assessment of tumors and to deliver extraordinarily powerful information for guiding patients' diagnoses, management and prognosis. Nonetheless, it must never be forgotten that the single most important determinant of diagnosis and management is the morphological assessment of tumors, complemented by the use of immunohistochemistry. Results of molecular testing cannot fully be dissociated from histopathological assessment and the results must be considered by pathologists, physicians, surgeons and oncologists, alongside the clinical information at multidisciplinary meetings. The advent of plasma-based tests makes this requirement an important arena.

As the repertoire of clinically relevant molecular alterations becomes ever larger, there is no doubt that molecular pathology reporting will become more and more complex. The aim will be to rationalize these extremely complex findings, including information about DNA, RNA, chromosomal structure and protein expression alterations in tumor cells and in tumor infiltrating lymphocytes into a clinically useful report which will effectively guide patient management. This requires not only technical and molecular pathological knowledge but also good understanding of histopathology and clinical medicine.

The technical challenges posed by the proliferation of molecular tests are also immense. As more treatments become available for increasingly advanced cancers, most specimens received by laboratories will increasingly be small biopsies and cytological specimens rather than large resections. Using this small amount of tissue, an exponentially increasing number of assays will need to be performed with ever shortening turnaround times. This will require not only a sensible approach to tests likely to gather useful information, but also a flexible approach and willingness to use multiple technological platforms to provide an efficient service.

We have seen a glimpse of the future in lung cancer, where extremely small biopsies require a bank of three molecular tests urgently:

1. EGFR mutation assessment using real-time PCR or, possibly using plasma, bearing in mind the issue of sensitivity.
2. ALK translocation tested using either IHC or FISH.
3. PD-L1 testing using any of a number of available IHC assays.

Increasingly, it is necessary to use second-line testing for numerous molecular alterations which may qualify patients for trials. To allow this information to be used in clinical practice, there is a need for sophisticated algorithms combining the results of multiplex testing involving both DNA and protein expression.

The challenges posed by this new endeavor are immense, and will require considerable funding, effort and ingenuity to implement. The reward, however, of potentially revolutionized cancer care and the prospect of significantly lengthened, high quality survival, could not be greater.

Acknowledgment

The authors acknowledge the use of some of the material and illustrations from the previous edition of this text.

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Useful websites

UKAS accreditation. https://www.ukas.com/services/accreditation-services/medical-laboratory-accreditation-iso-15189/

Examples of companies offering CE-IVD control material. https://www.horizondiscovery.com/. https://www.thermofisher.com/uk/en/home/brands/product-brand/acrometrix.html. https://www.seracare.com/.

Genomics England protocols. http://www.genomicsengland.co.uk/wp-content/uploads/2015/03/GenomicEnglandProtocol_030315_v8.pdf

Qiagen Qiacube, example of an automated spin column system. https://www.youtube.com/watch?v=egHqZqkLkAc.

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Qiagen pyrosequencing technology. https://www.qiagen.com/gb/resources/technologies/pyrosequencing-resource-center/technology-overview/. https://www.qiagen.com/gb/resources/e-learning/videos/pyrosequencing%20cascade%20reaction/.

HalioDX Immunoscore. www.haliodx.com

21 Transmission electron microscopy

Anthony E. Woods • John W. Stirling

Introduction

Transmission electron microscopy (TEM) is a significant tool in demonstrating the ultrastructure of cells and tissues both in normal and disease states. In particular, TEM can be crucial in the diagnosis of various renal pathologies, the recognition of subcellular structural defects or the deposition of extracellular material (e.g. in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, CADASIL) and in the typing of microsporidia. This chapter details the methods used to process and prepare tissue samples appropriately for examination in the transmission electron microscope (EM). The fundamental advantage of TEM over conventional light microscopy (LM) is that the EM has a resolution approximately 1000 times greater. With this increased resolving power, the EM is able to demonstrate the ultrastructure (substructure) of individual cells. Contemporary EMs with digital imaging systems are capable of resolving 0.2 nm (or less): using cryotechniques, this allows cell structures to be examined at the molecular level. However, in practical terms, biological tissues prepared using standard methods cannot be examined at such high resolution due to the limitations of chemical fixation and routine preparation techniques.

Tissue preparation for transmission electron microscopy

The basic preparation methods used in routine TEM are provided in this chapter. More detailed discussions of these, plus alternative and specialized procedures, can be found elsewhere (Glauert, 1972-1998; Robards & Wilson, 1993; Allen & Lawrence, 1994-1996; Glauert & Lewis, 1998; Hayat, 2000). A flow chart summarizing the steps required for preparing the basic range of diagnostic TEM specimens is given in Fig. 21.1.

The fundamental principle underlying TEM is that electrons pass through the section to give an image of the specimen. The electron beam is only capable of penetrating a resin section effectively to a depth of approximately 100 nm, so to obtain a high-quality image and optimize the resolution of the instrument, it is necessary to section the tissue to a thickness of around 80 nm.

Sectioning at this level requires tissues to be embedded in a rigid material which can withstand both the vacuum in the microscope column and the heat generated as the electron beam passes through the section. The wax embedding media used in LM are not suitable for this purpose and tissues must be embedded in an epoxy or acrylic resin. Both hydrophilic and hydrophobic media are available but, for routine purposes, a hydrophobic epoxy resin such as Araldite, Epon or Spurr's is preferred (see Chapter 8).

Specimen handling

In order to preserve the ultrastructure of a cell it is crucial that samples are fixed as soon as possible after the biopsy is taken. The most sensitive cellular indicators of autolytic/degenerative change are mitochondria and endoplasmic reticulum, both of which may show signs of swelling (a reflection of osmotic imbalance) only a few minutes after the cells are separated from a blood supply.

The standard approach is to immerse the specimen in fixative immediately on collection. Once in

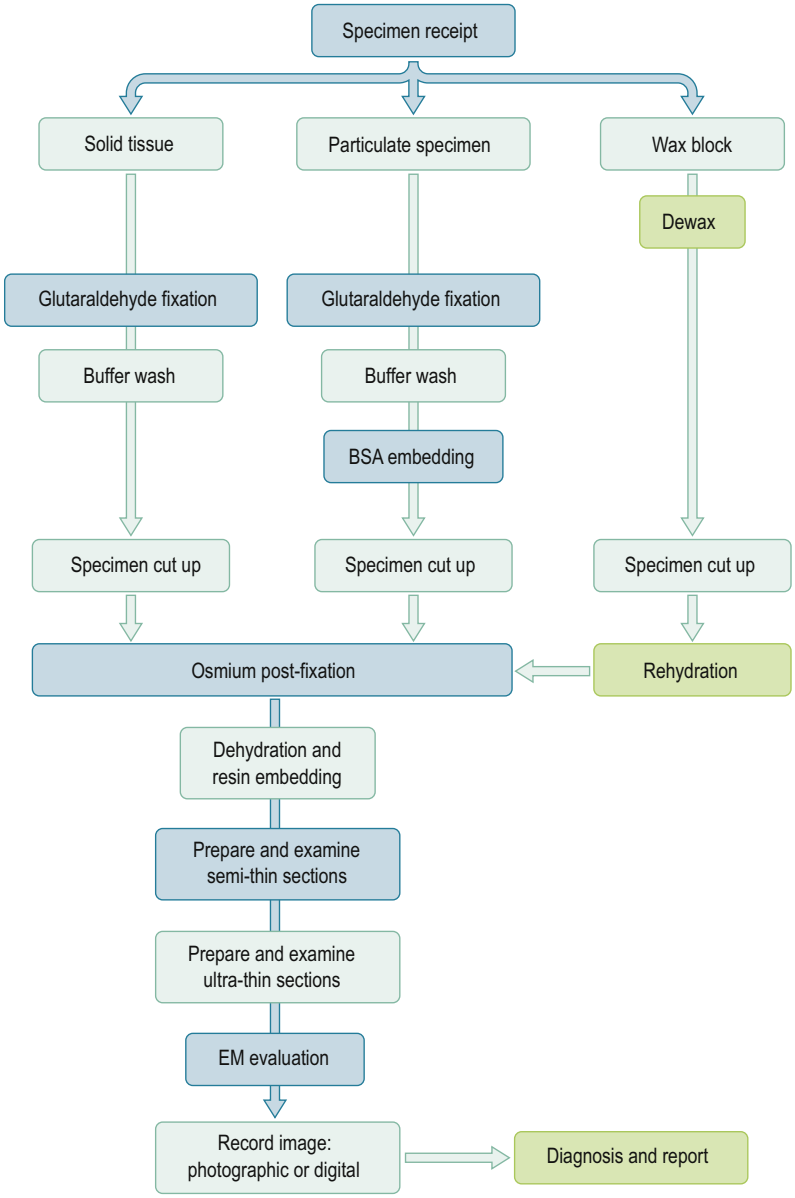


Fig. 21.1 Flow chart illustrating the major steps in the preparation of specimens for diagnosis by TEM. BSA, bovine serum albumen.

fixative, the specimen is cut into smaller samples using a scalpel or razor blade. At this point the tissue should be oriented and dissected to optimize exposure of the critical diagnostic features during sectioning and screening. Dissection must also facilitate the penetration of fixatives and processing reagents (Stirling, 2013a). The final tissue blocks may be in the form of thin sheets or small cubes (approximately 1 mm^3), although the risk of sampling error increases as the sample size decreases. In general, the volume of fixative should be at least 10 times the volume of the tissue. It is also vital to ensure that the tissue remains completely submerged in the fixative. One should be aware that small pieces may adhere to the

inside of the lid of the biopsy container, and therefore these will be poorly fixed even if they have been exposed to fixative vapor. Gentle agitation of the vial on a mechanical rotator helps to overcome this problem and improves fixation.

The importance of using small samples cannot be overemphasized. The use of cold fixative (4°C) helps to minimize postmortem changes but fixation may be hindered as a consequence. In addition, the penetration rate of most TEM fixatives is quite slow, increasing the risk of artifact formation. It should also be noted that fixatives and processing reagents penetrate different tissues at different rates. Some tissues, e.g. liver, fix poorly and needle biopsies may need to be cut longitudinally to ensure adequate fixation. If a delay in fixation is unavoidable, damage can be minimized by holding the tissue in chilled normal saline for a short time. However, the tissue must not be frozen at any point.

Fixation

The fixatives used in TEM generally comprise a fixing agent in buffer (to maintain pH) and, if necessary, various additives to control osmolarity and ionic composition. Other factors which affect fixation include fixative concentration, temperature, and the duration of fixation. The standard protocol involves primary fixation with an aldehyde, usually glutaraldehyde, to stabilize proteins, followed by secondary fixation in osmium tetroxide to retain lipids (Hayat, 1981), this is termed 'double fixation'.

Fixative concentration

Glutaraldehyde is effective at a concentration of between 1.5 and 4%, with 2.5% the simplest to prepare from commercially available 25% stock solutions. Osmium tetroxide is usually used at a concentration of 1 or 2%.

Temperature

Tissues may be placed in cold primary fixative solution, but this is not essential. Fixation at room temperature improves the penetration rate, particularly of aldehyde fixatives, and reduces the time required for fixation, although it may also increase the risk of autolytic change. Osmium tetroxide is generally used at room temperature.

Duration of fixation

The time required for optimal fixation depends on a range of factors. These include the type of tissue, the size of the sample, and the type of fixative and buffer system used. In most circumstances immersion of 0.5–1.0 mm^3 blocks of tissue in 2.5% glutaraldehyde fixative for 2–6 hours is sufficient. It is recommended that punch biopsies of skin taken for the diagnosis of CADASIL should be fixed overnight to ensure adequate preservation, particularly if they are left whole (i.e. not dissected) and sent to a distant laboratory for processing and screening (Stirling, 2013b). Secondary fixation in 1% osmium tetroxide for 60–90 minutes is usually effective but much longer times are required if osmium tetroxide is the primary fixative. The use of microwave irradiation can accelerate fixation times in aldehyde fixative to as little as 5–10 seconds (Leong, 1994), after which the sample may be stored in buffer or processed immediately.

Buffers

Fixatives are normally prepared in buffer (the fixative 'vehicle') which is adjusted within a range of pH 7.2–7.6 (Robinson & Gray, 1996). Ideally the osmolarity and ionic composition of the buffer should mimic that of the tissue being fixed. Generally this is not a major requirement but, an osmolarity slightly hypertonic to or equivalent of plasma (300-330 mOsm) is suitable for most circumstances. Non-ionic molecules such as glucose, sucrose or dextran are used to adjust tonicity as these will not influence the ionic constitution of the buffer. The addition of various salts, particularly calcium and magnesium, is thought to improve tissue preservation, possibly by stabilizing membranes (Hayat, 1981). This is unlikely to have a major effect in routine diagnostic applications.

Phosphate buffers

Phosphate buffers (Gomori, 1955) are the buffer of choice as they are non-toxic and work well with

most tissues but have two disadvantages which restrict their use. Firstly phosphate buffers are a good growth medium for molds and other microorganisms; secondly most metal ions form insoluble phosphates with this buffer. The phosphates of sodium, potassium and ammonium however, are soluble.

Phosphate buffer (0.1 M, pH 7.4)

Stock reagents

Solution a

Disodium hydrogen phosphate (Na_2HPO_4 anhydrous)	14.2 g
Distilled/deionized water	1000 ml

Solution b

Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$)	51.6 g
Distilled/deionized water	1000 ml

Method

Mix 40.5 ml of solution **a** with 9.5 ml of solution **b**. The pH should be checked and adjusted if necessary, using 0.1 M hydrochloric acid or 0.1 M sodium hydroxide.

Alternative buffers

Other buffers which have been recommended for use in TEM include cacodylate (Sabatini et al., 1963), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), MOPS (3-(*N*-morpholino) propanesulfonic acid) and PIPES (piperazine-*N*, *N*′-bis2-ethanesulfonic acid) (Good & Izawa, 1972; Massie et al., 1972; Salema & Brandão, 1973; Ferguson et al., 1980).

Aldehyde fixatives

Glutaraldehyde

Although glutaraldehyde is the most widely used primary fixative in TEM, its fixation reactions are poorly understood. The most important reaction of glutaraldehyde, stabilizing proteins, is thought to occur via a cross-linking mechanism involving the amino groups of lysine and other amino acids through the formation of pyridine intermediaries. Lipids and most phospholipids (those not containing free amino groups) are not fixed and will be extracted during subsequent processing without secondary fixation (Hayat, 2000).

Glutaraldehyde fixative (2.5%, buffered)

Stock reagents

25% glutaraldehyde stock solution	10 ml
0.1 M phosphate buffer, pH 7.4	90 ml

Method

Combine glutaraldehyde and phosphate buffer in the proportions indicated.

Formaldehyde

Commercially supplied formaldehyde solutions (formalin) normally contain some formic acid and considerable quantities of methanol and are poor cytological fixatives not suitable for TEM. In contrast, formaldehyde which has been freshly prepared from paraformaldehyde powder is adequate for TEM as it lacks impurities and also penetrates faster than glutaraldehyde. Paraformaldehyde has been used in conjunction with glutaraldehyde and may be useful for electron immunohistochemistry as tissue epitopes are less likely to be significantly altered during fixation and, if required, antigen unmasking is more effective.

Aldehyde combinations

The use of an aldehyde mixture may offset the disadvantages of glutaraldehyde (a slow penetration rate) and formaldehyde (less stable fixation) when applied individually (Karnovsky, 1965).

Paraformaldehyde (2%) and glutaraldehyde (2.5%) fixative (buffered) (based on Glauert, 1972; Karnovsky, 1965)

Stock reagents

0.2 M buffer, pH 7.4 (phosphate, cacodylate)	50 ml
Paraformaldehyde	2.0 g
25% aqueous glutaraldehyde	10 ml
Distilled/deionized water to	100 ml

Method

1. Completely dissolve paraformaldehyde in buffer using heat and with continuous stirring. It may be necessary to add a few drops of 1.0 M sodium hydroxide to clarify the solution.
2. Cool the solution rapidly under running water.
3. Add aqueous glutaraldehyde. Check the pH of the mixture and adjust if necessary to pH 7.4.
4. Add distilled water to make 100 ml.

Note

Adding 0.2 ml of 1.0 M calcium chloride is thought to have a membrane stabilizing effect but this may precipitate if phosphate buffer is used.

Osmium tetroxide

The use of osmium tetroxide fixation to preserve lipids is fundamental to TEM (Palade, 1952; Millonig & Marinozzi, 1968). Whilst primary fixation in osmium tetroxide is effective, its extremely slow penetration rate can give rise to autolytic changes. For this reason, osmium tetroxide is usually used as a secondary fixative, termed 'post-fixation', after primary fixation in aldehyde. The penetration rate of osmium tetroxide is also higher in stabilized tissue and immersion for 60–90 minutes is sufficient for most specimens.

Osmium tetroxide is usually supplied in crystalline form, sealed in glass ampoules. Extreme care should be exercised when preparing this material, gloves and eye protection should always be worn. It is essential to only handle osmium tetroxide in a fume-hood, as the vapor will also fix other tissues, including the eyes and nasal tissues of the handler.

Specimens fixed in aldehyde solutions should be washed thoroughly in buffer before post-fixation in osmium tetroxide to prevent interaction between the fixatives which can cause precipitation of reduced osmium. Osmium tetroxide can be prepared as an aqueous solution, although it can also be made in the same buffer used to prepare the primary fixative. Osmium tetroxide should be avoided if electron immunogold labeling studies are to be performed, as it has the potential to alter protein structure significantly, rendering epitopes unreactive.

Osmium tetroxide fixative (2% aqueous)

Stock reagent

Osmium tetroxide	1.0 g
Distilled/deionized water	50 ml

Method

1. In a fume-hood, clean then score the glass ampoule with a diamond pencil and place in a dark glass storage bottle.
2. Break the ampoule with a glass rod and add water. It may take 24 hours or longer for the osmium to dissolve completely unless ultrasonicated.
3. Prepared solutions may be stored for short periods at room temperature in the dark in a well-sealed bottle (double wrap the bottle in aluminum foil); for long periods store at 4°C. All containers of osmium solutions should be stored inside a second well-sealed container to prevent the leakage of osmium fumes; milk powder can be placed in the second container to absorb fumes which leak from the primary container. Aqueous osmium solutions which are prepared and stored in a clean container should last for approximately 1 year; solutions in buffer may only last a few days before they deteriorate.
4. For a 1% working solution combine 1:1 with water or buffer.

Note

Osmium is readily reduced by dust and light. Only glassware which has been acid cleaned and thoroughly rinsed in distilled water should be used. Prepared solutions should be monitored during storage and discarded if a pink color develops.

Wash buffer and staining

After primary fixation in glutaraldehyde, tissue may be treated in several ways. Material which is to be retained may be rinsed briefly in a buffer compatible with the fixative vehicle, then stored in fresh buffer. Tissue for immediate processing should be washed in buffer before post-fixation in osmium tetroxide then washed again in buffer or water to remove excess osmium. This is critical as osmium tetroxide and alcohol react to form a black precipitate. An optional step at this point is to immerse tissues after post-fixation in 2% aqueous uranyl acetate. This en bloc staining procedure adds to the contrast of the final sections and improves preservation. However,

it should be noted that uranyl acetate can extract glycogen.

Dehydration

The most common embedding compounds used in TEM are epoxy resins. These are immiscible with water and specimens must be dehydrated prior to resin infiltration. Dehydration is performed by passing the specimen through increasing concentrations of an organic solvent. It is necessary to use a graded series to prevent the damage which would occur with extreme changes in solvent concentration. It is also important to keep the dehydration times as brief as possible to minimize the risk of extracting cellular constituents. The most frequently used dehydrants are acetone and ethanol, but methanol may also be used (Stirling, 2013a). Acetone should be avoided if en bloc staining with uranyl acetate has been performed to prevent precipitation of uranium salts. Ethanol overcomes this difficulty but requires the use of propylene oxide (1,2-epoxypropane) as a transition solvent to facilitate resin infiltration. Residual dehydrant can result in soft or patchy blocks.

Commercially available 'absolute' ethanol normally contains a small percentage of water. This will severely restrict infiltration and polymerization of the resin and it is necessary to complete dehydration in anhydrous ('super dry') ethanol obtained commercially or prepared by using an appropriate molecular sieve. Propylene oxide is highly volatile, flammable and may form explosive peroxides. It should be stored at room temperature in a flammable solvents safe.

Embedding

After dehydration (and, if required, treatment with a transitional solvent) the tissue is infiltrated with liquid resin. The resin is introduced gradually, beginning with a 50:50 mix of transition solvent (propylene oxide) and resin followed by a 25:75 mix, then finally pure resin. An hour in each of the preliminary infiltration steps is usually adequate, although some recommend leaving samples in pure resin for 24 hours. Gentle agitation using a low-speed, angled rotator during these steps will assist resin infiltration. McDonald (2014) used centrifugation for rapid infiltration with epoxy and acrylic resins and it is possible that this novel technique may be adapted for routine samples. Failure to completely infiltrate the tissue with resin will cause major sectioning difficulties.

Once infiltrated, tissue samples are placed in an appropriate capsule or mold (various shapes and sizes are available) which is filled with resin. A paper strip bearing the tissue identification code written in pencil or laser-printed should be included with each sample. The resin is polymerized using heat. Soft polyethylene capsules (resistant to 75°C) are recommended for general embedding, but for high temperature embedding use hard polypropylene capsules (resistant to 100°C). The block is released by cutting away the capsule with a razor blade or scalpel. Flat embedding molds made of silicone rubber can be used to make rectangular blocks, these are removed by bending the mold, which can potentially be reused.

Epoxy resins

Epoxy resins have been the embedding medium of choice in TEM since their introduction in the mid-1950s (Glauert et al., 1956). The main types are Araldite (Glauert & Glauert, 1958), Epon (Luft, 1961) and Spurr's resin (Spurr, 1969). Although the original product names Araldite and Epon refer to epoxy resins developed by the CIBA Chemical Company and Shell Chemical Company respectively, these terms are now in general use. During polymerization, epoxy resins form cross-links, creating a three-dimensional polymer of great mechanical strength. As well as their properties of uniform polymerization and low shrinkage (usually less than 2%), epoxy resins also preserve tissue ultrastructure, are stable in the electron beam, section easily and are readily available.

Epoxy resins usually comprise four ingredients: the monomeric resin, a hardener, an accelerator and a plasticizer. Manufacturers generally provide a standard formulation, however the hardness and flexibility of the polymerized block can be manipulated by varying the amount of the individual components. Correspondingly, it is critical to measure components (either by volume or weight) carefully. The simplest approach is to weigh the components into a disposable paper or plastic cup as unused resin can be polymerized and discarded in the container. Thorough mixing of the components is absolutely essential. When

prepared, the resin is best delivered through a non-reactive plastic syringe or pipette.

Occupational exposure to epoxy resins is a common cause of allergic contact dermatitis (Kanerva et al., 1989; Jolanki et al., 1990). These agents are also probable carcinogens, primary irritants and systemically toxic (Causton, 1981), and therefore should be handled with care.

Acrylic resins

Acrylic resins (methacrylates) derive from methacrylic acid [CH_2=C·(CH_3)COOH] and acrylic acid [CH_2=CH·COOH] and were the original synthetic media developed for use in TEM. Acrylic resins can rapidly infiltrate fixed, dehydrated tissues at room temperature. However, marked variable shrinkage of tissue components was common due to unreliable polymerization and early acrylic resins proved unstable in the electron beam. Acrylic monomers are of low viscosity, and both hydrophilic and hydrophobic forms are available. Acrylic resins react by free radical polymerization, which can be initiated using light, heat or a chemical accelerator (catalyst) at room temperature.

The main commercial acrylic resins are LR White, LR Gold, and the Lowicryl series (K4M, K11M, HM20 and HM23). Each of these can be used for low-temperature dehydration and embedding to reduce the heat damage from exothermic polymerization and extraction by solvents and resin components (Acetarin et al., 1986; Newman and Hobot, 1987, 1993). These characteristics make several forms of acrylic resin ideally suited to electron immunogold labeling (Stirling, 1994) and enzyme cytochemical studies (see Chapter 8).

Tissue processing schedules

Manual tissue processing is best performed by keeping the tissue sample in the same vial throughout, and using a fine pipette to change solutions. When processing multiple samples, take care not to cross-contaminate specimens by using separate pipettes. All vials must be clearly labeled and labels must be 'solvent-proof'. It is advantageous to gently agitate tissue specimens on an angled rotator throughout the processing cycle to enhance reagent penetration. A protocol for the routine processing of solid tissue samples is given in Table 21.1.

Table 21.1 Standard processing schedule for solid tissue cut into 1 mm^3 blocks (each step is performed at room temperature unless stated otherwise)

Primary fixation	2.5% glutaraldehyde in 0.1 M phosphate buffer	2–24 hours (room temperature or 4°C)
Wash	0.1 M phosphate buffer	2 × 10 minutes on rotator
Post-fixation	1% aqueous osmium tetroxide	60–90 minutes
Wash	Distilled water	2 × 10 minutes
En bloc staining (optional)	2% aqueous uranyl acetate	20 minutes
Dehydration	70% ethanol	10 minutes on rotator*
	90% ethanol	10 minutes on rotator
	95% ethanol	10 minutes on rotator
	100% ethanol	15 minutes on rotator
	Super dry absolute ethanol	2 × 20 minutes on rotator
Transition solvent (clearing)	1,2-epoxypropane	2 × 15 minutes on rotator
Infiltration	50:50, clearant:resin#	1 hour on rotator
	25:75, clearant:resin	1 hour on rotator
	Resin only	1–24 hours (with vacuum to remove bubbles)
Embedding	Fresh resin in embedding capsules	12–24 hours at 60–70°C

*Tissues may be stored at this stage.

#As batches may vary, resin should be prepared in accordance with manufacturer's instructions.

Procedures for other tissue samples

Cultured cells

Cell cultures may be fixed in situ, then separated from the substrate, centrifuged into a pellet and treated as a solid tissue. Alternatively, cells can be harvested into a centrifuge tube and processed as a suspension (see below) or pelleted lightly, re-suspended in fixative and again pelleted by gentle centrifugation. After fixation the pellet can be cut into cubes for further processing. Cell cultures may also be fixed and processed whilst attached to the substratum, then inverted embedding capsules are pressed onto the cell layer. Once polymerized, blocks can be separated by force or after being cooled in liquid nitrogen (see 'Pop-off' technique, below).

Cell suspensions or particulate matter

Cell suspensions (e.g. fine needle biopsy aspirates, bone marrow specimens or cytology samples) or particulate materials (including fluid aspirates, tissue fragments or products and specimens for the assessment of ciliary structures) are best embedded in a protein support medium before processing. Plasma, agar or bovine serum albumen (BSA) can be used. The addition of tannic acid (Hayat, 1993) during the preparation of ciliary specimens gives improved visualization of axonemal components (Sturgess & Turner, 1984; Glauert & Lewis, 1998). The tannic acid is thought to act as a fixative and a mordant, facilitating the binding of heavy metal stains (Hayat, 2000). Double en bloc staining with uranyl acetate and lead aspartate may also improve the visibility of dynein arms (Rippstein et al., 1987).

Preparing particulate samples

Stock reagents

15% aqueous BSA

0.1% tannic acid (low MW) in pH 7.4 phosphate buffer

Method

1. Centrifuge the sample in buffer in a plastic centrifuge tube to form a loose pellet.
2. Discard supernatant and re-suspend the sample in glutaraldehyde fixative at room temperature for a minimum of 1 hour.
3. Centrifuge the sample and carefully discard the supernatant.
4. Wash the sample by re-suspending in buffer for 10–15 minutes.
5. Centrifuge the sample to form a loose pellet.
6. Discard supernatant and introduce 0.5 ml of 15% aqueous BSA. Re-suspend the specimen and allow BSA to infiltrate for a minimum of 1 hour.
7. Centrifuge the sample and discard most of the supernatant, leaving sufficient to cover the pellet to a depth of approximately 1 mm.
8. Carefully introduce an equal volume of glutaraldehyde fixative to form a layer above the BSA. Allow sample to solidify for 2–24 hours.
9. Remove the sample (this is most easily achieved by cutting away the plastic centrifuge tube) and divide into small portions.
10. Wash in four changes of buffer, each for 5 minutes (for ciliary biopsies only, incubate for 15 minutes in buffered tannic acid solution, then wash in four changes of buffer, each for 5 minutes, before proceeding to step 11).
11. Post-fix in 1% aqueous osmium tetroxide and process as normal.

Material embedded in paraffin wax/cell smears

Cell smears and paraffin wax-embedded tissue for LM can be reprocessed for TEM. As the quality of preservation may vary considerably, care must be exercised in the ultrastructural interpretation of such material. Nevertheless, it is often possible to obtain sufficient, or additional, diagnostic information.

Reprocessing paraffin wax-embedded material

Method

1. Remove the area of interest from the block, taking care not to damage the tissue.
2. Dewax the specimen by passing through several changes of xylene. The time required depends on the size of the sample but should be at least 1 hour. A minimum of three changes is recommended.
3. Rehydrate the material in a graded ethanol series.
4. Wash in water, post-fix in osmium tetroxide and process as a routine specimen (see above).

Pop-off technique for slide-mounted sections (after Bretschneider et al., 1981)

Method

If additional fixation is required

1. Remove the coverslip by soaking the slide in xylene. (This may take some time. An alternative is to firstly cool slides to –20°C for up to one hour, then carefully remove the coverslip with a blade.)
2. Rehydrate the tissue in a graded ethanol series.
3. Wash in buffer and fix the tissue in glutaraldehyde fixative for 15–20 minutes.
4. Wash in buffer and post-fix in 1% osmium tetroxide for 20–30 minutes.
5. Wash in buffer or distilled water, and then cover with 2% uranyl acetate for 15 minutes.
6. Dehydrate the tissue by passing the slide through 70%, 90%, 95%, 100% and super dry ethanol for 5 minutes in each stage.
7. Dip the slide into propylene oxide for 5 minutes. The tissue should not be allowed to dry.
8. Cover the tissue with a 2:1 mixture of propylene oxide and epoxy resin for 5–15 minutes. The tissue should not be allowed to dry.
9. Cover the tissue with a 1:2 mixture of propylene oxide and epoxy resin for 5–15 minutes. The tissue should not be allowed to dry.
10. Cover the tissue with full-strength epoxy resin for 5–15 minutes.
11. Drain off surplus resin mixture. Invert a freshly filled (to overflowing) embedding capsule over the section and press onto the slide.
12. Incubate the slide and capsule at 60°C for 24 hours for polymerization to occur.
13. Remove the slide and, whilst still warm, separate the capsule and the newly embedded tissue from the glass slide.

If additional fixation is not required

1. Remove the coverslip by soaking the slide in xylene. (This may take some time. An alternative is to firstly cool slides to –20°C for up to one hour, then carefully remove the coverslip with a blade.)
2. Dip the slide in equal parts of propylene oxide and xylene, then into propylene oxide for 5–10 minutes. The tissue should not be allowed to dry.
3. Cover the tissue with a 2:1 mixture of propylene oxide and epoxy resin for 5–15 minutes. The tissue should not be allowed to dry.
4. Cover the tissue with a 1:2 mixture of propylene oxide and epoxy resin for 5–15 minutes. The tissue should not be allowed to dry.
5. Cover the tissue with full-strength epoxy resin for 5–15 minutes.
6. Drain off surplus resin mixture. Invert a freshly filled (to overflowing) embedding capsule over the section and press onto the slide.
7. Incubate the slide and capsule at 60°C for 24 hours for polymerization to occur.
8. Remove the slide and, whilst still warm, separate the capsule and the newly embedded tissue from the glass slide.

Note

Sections or cell cultures are easier to prepare using the pop-off method if mounted or grown directly on Thermanox coverslips. Thermanox coverslips are made of a proprietary polystyrene-like compound which is resistant to fixatives and common solvents and separates easily from the face of resin blocks.

Microwave processing

Microwaves can be applied to speed up individual processing steps in TEM or to the entire processing schedule. A full microwave protocol allows the rapid turnaround of tissues within approximately 5 hours for 'same-day' diagnostic reporting, or to approximately 3 hours 20 minutes for extremely urgent samples (Schroeder, 2013). Commercial semi-automatic and fully automatic microwave tissue processors are available and processing schedules are outlined by Schroeder (2013). Additional information on microwave processing for TEM can be found in Webster (2014).

Ultramicrotomy

Glass knives

Knives are prepared from commercially available plate glass strips manufactured specifically for ultramicrotomy. Before use, the strips should be cleaned by washing thoroughly with detergent, rinsed in distilled water and alcohol then dried using lint-free paper. Most knife makers will allow

knives of different cutting edge angles to be produced. Higher angle knives (up to 55°) are best suited to cutting hard materials, whilst softer blocks respond better to shallower (35°) angle knives. Glass squares and knives should be prepared just before use to avoid contamination and stored in a dust-free container.

Knives should always be inspected before use. If the knife edge is correctly formed, when it is observed face-on, it should be straight and even but with a small glass spur on the top right-hand end (Fig. 21.2). The edge need not be horizontal, but those which are obviously convex or concave should be discarded. The knife should also display a conchoidal fracture mark which curves across and down from the top left-hand edge of the knife until it meets the right-hand edge of the glass. Each of these characteristics is visible macroscopically. When placed in the ultramicrotome and viewed under the microscope the cutting edge will appear as a bright line against a dark background. The left third of the cutting edge should appear as a smooth line and is the zone recommended for thin sectioning. The middle third is often adequate but can show minute imperfections so this area is best reserved for trimming blocks prior to sectioning and for cutting semi-thin sections.

In ultramicrotomy, thin sections are floated out for collection as they are cut. This requires a small trough to be attached directly to the knife. Pre-formed plastic or metal troughs which can be fitted to the back of the knife are commercially available. These may need to be sealed with molten dental wax or nail varnish after attachment, but they are expedient and simple to use. An alternative approach is to prepare a trough using self-adhesive PVC or polyester tape, sealing the lower edge of the trough with molten dental wax (Fig. 21.3).

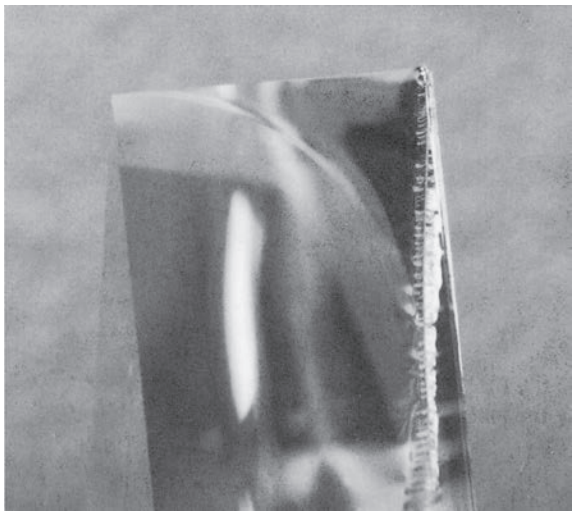


Fig. 21.2 Glass knife prepared from 6.4 mm thick glass strip. Note the straight cutting edge and conchoidal fracture mark.

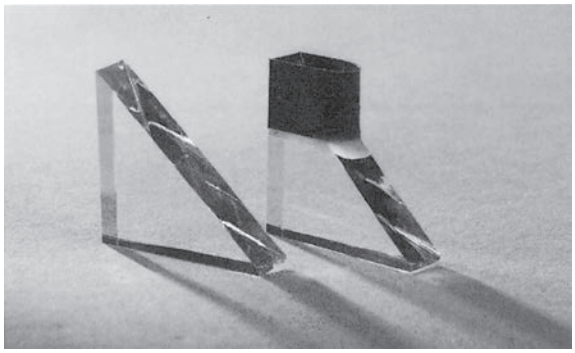
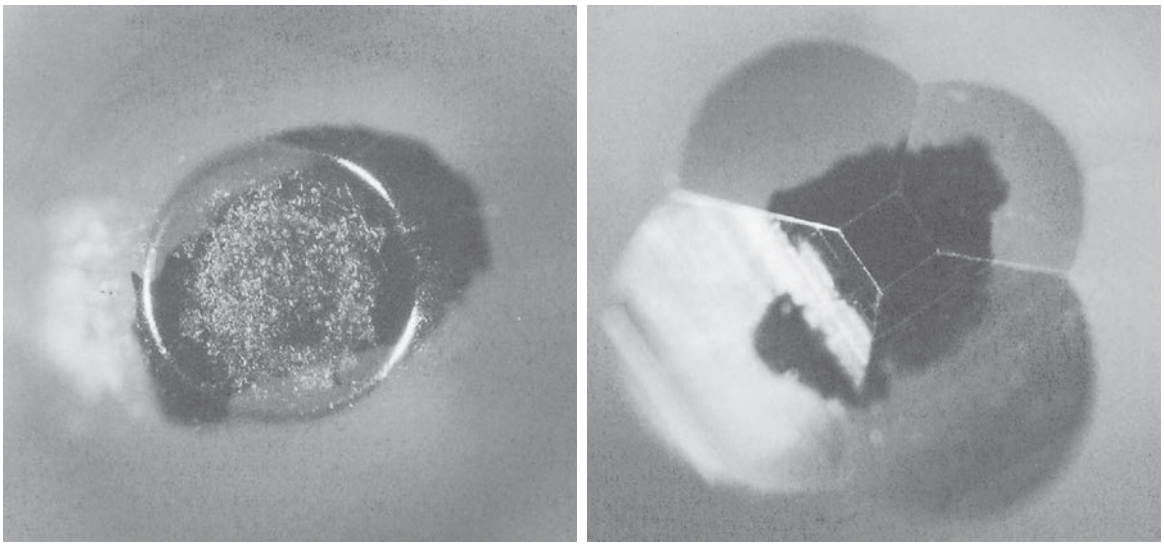


Fig. 21.3 Glass knives: left, bare knife used for trimming and semi-thin sectioning; right, knife used for ultra-thin sectioning with a fitted trough prepared from plastic (PVC) tape.

Diamond knives

A well-maintained diamond knife is capable of cutting any type of resin block, most biological and many non-biological materials except those containing hard inclusions (see Chapter 8). Commercial knives are mounted in a metal block incorporating a section collection trough designed to fit directly into the knife holder of the ultramicrotome. Diamond knives are brittle but durable and will continue to cut for quite some time provided they are kept clean and treated carefully. Commercially available polystyrene cleaning strips can be used to clean along, never across, the cutting edge. A diamond knife must only be used to cut ultra-thin sections. It should not be used 'dry' without trough fluid, or to cut semi thin sections, unless it has been designed for these purposes. Diamond knives can be professionally sharpened when worn or damaged.



a b

Fig. 21.4 Araldite blocks **(a)** untrimmed **(b)** trimmed of excess resin.

Trough fluids

The simplest and most suitable fluid routinely used for section collection is distilled or de-ionized water; 10–15% solutions of ethanol or acetone can also be used but for a diamond knife, check that the mounting is resistant to the solvent being used. It is important to ensure that the correct level of fluid is added. If the level is too high, the fluid will be drawn over the cutting edge and down the back of the knife, preventing proper sectioning. If the level is too low, sections will accumulate on the cutting edge and will not float out.

Block trimming

Once polymerized, blocks must be cleared of excess resin (trimmed) to expose the tissue for sectioning. The final trimmed area should resemble a flat-topped pyramid with a square or trapezium-shaped face (Fig. 21.4).

Trimming the block can be achieved manually or by using the ultramicrotome. Manual trimming can be performed by mounting the block in a suitable holder under a dissecting microscope and removing the surplus resin with a single-edged razor blade. Although this method is quite speedy, considerable care is required to ensure the ultimate cutting surface is as level as possible to facilitate sectioning. Alternatively, the block is positioned in the ultramicrotome and mechanically trimmed using a glass knife.

Semi-thin sections

Semi-thin (or 'survey') sections allow samples to be screened by LM for specific features and to select areas for thin sectioning. Semi-thin sections are usually cut on a glass knife, but there are now diamond knives specifically produced for this purpose.

Commonly, semi-thin sections are cut at between 0.5 and 1.0 μm from trimmed or partly trimmed blocks using the ultramicrotome and a glass knife. Sections can be cut dry using a slow cutting speed then picked up with forceps directly, or cut wet onto flotation fluid. Sections are transferred to a drop of water on a glass microscope slide and dried on a hot plate at 70–80°C. Semi-thin sections can be examined using phase contrast, or be stained and viewed by bright-field microscopy. Various cationic dyes, including methylene blue, azure B (Richardson et al., 1960) and crystal violet can be used for this purpose, although the most common is toluidine blue with borax. All are applied at high alkaline pH and with heat to facilitate penetration through the resin.

Toluidine blue stain for semi-thin sections

Stock reagents

Sodium tetraborate (borax)	1 g
Toluidine blue	1 g
Distilled/deionized water to	100 ml

Dissolve the borax in the distilled water and then add the toluidine blue. After filtering, the final solution can be stored at room temperature.

Method

1. Cover sections with staining solution and heat on a hot plate at 70–80°C.
2. Allow to stain adequately (the time is not crucial – up to 60 seconds is usually sufficient), then wash thoroughly in running water. Allow section to dry.
3. Sections can be viewed dry or mounted in DPX or epoxy resin.

Note

Borax raises the pH of the final stain to around pH 11.

Section collection

Ultra-thin sections are collected on specimen grids for viewing. Grids measure 3.05 mm in diameter and are made of conductive material, commonly copper, nickel or gold. Silver, palladium, molybdenum, aluminum, titanium, stainless steel and nylon-carbon are available, as well as combinations of the above. A large range of patterns and mesh sizes are available (Fig. 21.5), with 200 square mesh being commonly used, although slotted, parallel bar and hexagonal patterns are also standard. The choice of grid is a compromise between support for the sections (better with grids of smaller mesh size) and the relative proportion of exposed section (better with grids of larger mesh size). The latter provides a large area of section for viewing but with less stability. Copper grids (or coated copper grids) are generally used for routine TEM. Nickel grids, commonly used for immunolabelling studies, are not recommended for general use as they gain electric charge easily and can cause astigmatism.

Support films

The use of support films is generally unnecessary for routine epoxy resin sections. If, however, larger viewing areas are required, it may be necessary to use support films to provide greater section stability.

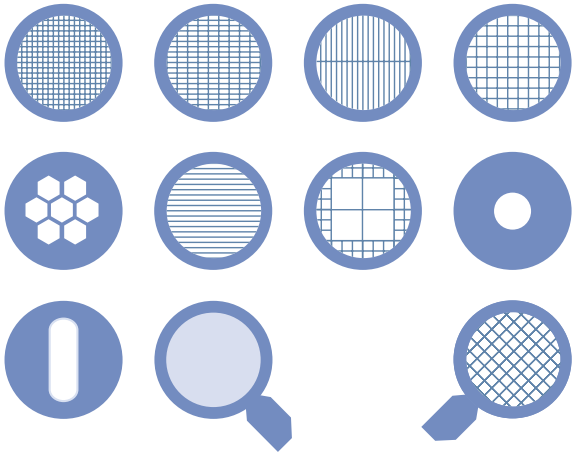


Fig. 21.5 Some examples of specimen grids. From top left: mesh (200 size); slotted (200 size); parallel with divider (200 size); mesh (50 size); hexagonal (7 size); parallel (75 size); freeze fracture; single hole; slotted; tabbed mesh (400 size); tabbed mesh (75 size).

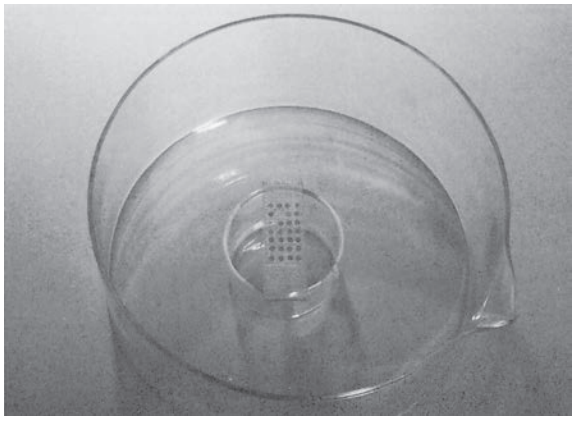


Fig. 21.6 Apparatus for application of plastic support films. The water level is raised over the level of the wire mesh, on which grids are then placed. Approximately 0.2 ml of liquid plastic film is dropped onto the water surface over the submerged grids and the solvent allowed to evaporate. The water is then drawn off, allowing the film of plastic to settle onto the grids.

Electron-transparent plastic films prepared from collodion, Formvar, Pioloform or Butvar are commonly used. There are many methods for applying plastic films; a simple method is illustrated in Fig. 21.6. Commercially prepared coated grids are available from specialist suppliers.

Ultra-thin sectioning

The basic principles of ultramicrotomy are similar, regardless of the ultramicrotome used (Reid, 1975;

Dykstra, 1992). Difficulties with sectioning usually relate to tissue which is poorly fixed or inadequately infiltrated and/or an imperfectly polymerized block or a dull knife (Table 21.2). Specific instructions on operating particular ultramicrotomes are normally provided by the instrument manufacturer.

A key element in ultramicrotomy is to ensure the sections are cut at a thickness which allows optimal resolution and specimen contrast. The most effective estimate of section thickness is given by viewing the interference color reflected from the section as it is cut. This color is the result of interactions between light waves reflected from the upper and lower surfaces of the section and is directly related to section thickness (Table 21.3).

Silver to gold sections (around 80 nm) are recommended. Thinner sections will give improved

Table 21.2 Causes and remedies for common sectioning faults

Fault	Effect	Potential cause	Remedy
Scoring	Scratches, tears in section running perpendicular to cutting edge	Knife edge damaged or dirty Hard material in block	Use a new section of knife Replace knife Use diamond knife
Sections contaminated	Artifacts, dirt on section	Dirt on knife edge Dirty trough fluid Trough dirty Block face dirty Dirty grids/forceps	Replace knife Replace trough fluid Replace with new knife and trough Trim and re-face block Clean appropriately
Chatter	Periodic variations in part (or all) of the section running parallel to the cutting edge (venetian blind effect)	Vibrations in knife, block or block holder on ultramicrotome Dull knife Block soft or unevenly polymerized Cutting speed too fast Clearance angle too great	Tighten components Replace knife Re-incubate block (60–70°C) for up to 24 hours Modify processing schedule and/or resin formulation Reduce cutting speed Reduce clearance angle
Compression	Specimen distortion with compression in the direction parallel to the cutting edge and extension in the direction perpendicular to the cutting edge	Block soft Sections too thin Cutting speed too fast Cutting edge angle too high	Re-incubate block (60–70°C) for up to 24 hours Modify processing schedule and/or resin formulation Increase section thickness Reduce cutting speed Prepare knife with shallower cutting edge angle
Section wrinkling or folding	Electron-dense bands with straight sides but of variable width	Block soft or unevenly polymerized Dull knife Block face too large Knife angle too shallow Section collection technique poor Picking up single sections	Re-incubate block (60–70°C) for up to 24 hours Modify processing schedule and/or resin formulation Replace knife Trim to a smaller block face Increase knife angle Improve technique Use ribbons

Table 21.2 Causes and remedies for common sectioning faults—cont'd

Fault	Effect	Potential cause	Remedy
Alternating thick and thin sections	Only some sections useful	Block face too large Incorrect knife angle Cutting speed too fast Dull knife Block soft or unevenly polymerized Vibration in ultramicrotome Air movement over sections during cutting	Trim to a smaller block face Adjust knife angle Reduce cutting speed Replace knife Re-incubate block (60–70°C) for up to 24 hours Modify processing schedule and/or resin formulation Tighten components Eliminate air drafts
Failure to cut sections as ribbon	Single sections	Upper and lower block edges not parallel Upper and lower block edges not straight Fluid level in trough too high or too low Cutting speed too slow	Re-trim block face Re-trim block face Adjust fluid level Increase cutting speed
Skipping (sections cut on alternate strokes)	Single sections	Dull knife Clearance angle too high Knife angle too high	Replace knife Reduce clearance angle Reduce knife angle

resolution but may not provide sufficient contrast for adequate on-screen viewing. Although it is possible to collect and examine individual sections, ribbons are easier to manage, and as the sections are in series they usually offer additional morphological information. Before collection, sections should be flattened to remove wrinkles, using heat, or ether, chloroform, xylene or amyl-acetate vapor. Vapors are applied using a sharpened orange-wood stick which is soaked in the fluid of choice, then held over the sections. Do not allow the stick to come into contact with the flotation fluid as the sections may be spoiled. Heat is best applied using a fine hot wire.

To collect sections, immerse the grid in the flotation fluid, then position it under the ribbon. Sections may be maneuvered whilst they are floating on the knife bath using a fine hair mounted on an orange-wood stick. If the grid is then angled slightly, the ribbon of sections will fall across the diameter of the grid as it is lifted from the fluid. It is important to remove any remaining fluid by gently touching the grid against lint-free absorbent paper to ensure the sections become firmly fixed. Clean, fine-point forceps should always be used to hold the grid. This requires great care to avoid damaging the grid either before or after collecting the sections. After collecting the sections, grids should be placed on filter paper in a lidded

Table 21.3 Relation between section thickness and interference color

Color	Section thickness (nm)
Gray	<60
Silver	60–90
Gold	90–150
Purple	150–190
Blue	190–240

container, such as a Petri dish, and allowed to dry completely before staining. The specimens should be clearly labeled on the filter paper. As they are fragile, it is strongly recommended that grids are stored in a dust-free grid storage box or in gelatin capsules. This protects them and provides a means of identifying individual grids.

Staining

Image contrast is a function of the accelerating voltage of the electron beam, the size of the objective aperture, section thickness and section staining. The purpose of staining is to increase the ability of cellular structures to scatter electrons, i.e. increase their electron density, thus contributing to specimen contrast. This is achieved by introducing heavy metal atoms which deposit on the various tissue components. Note that during specimen examination in the EM, contrast can also be manipulated by changing the size of the objective aperture (decreasing the diameter of the aperture increases the contrast), or manipulated electronically if the microscope has a digital imaging system.

Tissues can be stained at several points during preparation (Glauert & Lewis, 1998; Hayat, 2000):

- During secondary fixation, as osmium is deposited in membranes.
- When uranyl acetate is used during the post-fixation wash.
- By staining the sections with lead and uranium salts, known as en section staining.

The standard method for staining sections is to float the grids, section-side-down, on drops of stain solution. Alternatively, grids may be completely immersed in the solution. The procedure is carried out on a clean surface to minimize contamination. Normally, sections are stained in uranyl acetate followed by lead citrate (Reynolds, 1963). After each staining step, the grid is washed under a gentle stream of distilled water or by dipping it in distilled water. Finally, the grids are dried using clean lint-free filter paper. If the level of contrast achieved is not sufficient, a double lead staining method can be used (Daddow, 1983).

Uranyl salts

Uranyl acetate is the uranium salt normally used in TEM, although uranyl nitrate and magnesium uranyl acetate are also effective. The uranyl ions combine in large quantities with phosphate groups in nucleic acids, as well as phosphate and carboxyl groups on the cell surface (Hayat, 2000). Aqueous solutions of between 2 and 5%, applied to the section will give satisfactory contrast, but more intense staining can be achieved in less time by using a saturated ethanolic (or methanolic) solution (approximately 7%). Uranyl acetate is radioactive and highly toxic, its effects are cumulative and appropriate safety precautions should be followed.

Uranyl acetate (2% aqueous)

Stock reagents

Uranyl acetate	2.0 g
Distilled/deionized water	100 ml

Combine reagents in proportions indicated. Filter, divide into suitable aliquots and store at 4°C in the dark. Centrifuge before use.

Method

1. Place droplets of the staining solution on a clean surface in a lidded Petri dish (a fresh piece of Parafilm® M provides a suitable clean hydrophobic surface).
2. Place grid, section side down on the stain droplet for up to 10 minutes.
3. Rinse grids in three changes of distilled water.

Lead salts

Lead stains increase the contrast of a range of tissue components but must be prepared and used carefully as lead ions react with atmospheric carbon dioxide to form a fine precipitate of lead carbonate. This deposit appears as an electron-dense contaminant on sections and it cannot be removed easily. The commonly used method of Reynolds (1963) addresses this problem by chelating, and thus shielding, the lead ion from exposure to the carbon dioxide.

Reynolds' lead citrate stain (Reynolds, 1963)

Stock reagents

Lead nitrate	2.66 g
Trisodium citrate	3.52 g
1 M sodium hydroxide (freshly prepared)	16.0 ml
Distilled/deionized water (freshly prepared, carbonate-free)	84.0 ml

Mix the solid reagents in an alkaline-cleaned stoppered flask with approximately 60 ml of the water, inverting continuously for 1 minute. Allow to stand for 30 minutes with occasional mixing. Add the sodium hydroxide and mix until the solution becomes clear. Make up to 100 ml with remaining water. Divide into suitable aliquots and store at 4°C. Centrifuge before use.

Method

1. Place droplets of the staining solution on a clean surface in a lidded Petri dish (a fresh piece of Parafilm® M provides a suitable clean hydrophobic surface). The dish should also contain a few pellets of sodium hydroxide (to absorb carbon dioxide).
2. Place grid, section-side-down, on the stain droplets for up to 10 minutes.
3. Rinse grids in three changes of distilled water.

Diagnostic applications

Here we describe only the essential features of selected diseases in which TEM plays a major diagnostic role. For in-depth analyses there are a large number of specialist texts which contain a wealth of information on the interpretation of ultrastructural morphology. For example, the ultrastructural pathology of the cell has been covered by Ghadially (1997), non-neoplastic diseases by Papadimitriou et al. (1992b) and neoplastic diseases by Henderson et al. (1986), Erlandson (1994) and Ghadially (1985). Renal disease is comprehensively described by Zhou et al. (2009). Resources on renal pathology are also available online including, e.g. the American Journal of Kidney Diseases Atlas of Renal Pathology II at http://www.ajkd.org/content/atlasofrenalpathologyii (viewed November 2017). A comprehensive guide to diagnostic TEM (interpretation and technique) is given in Stirling et al. (2013b).

The use of TEM for diagnostics

TEM is used to obtain structural and compositional information which cannot be acquired using an alternative technique. In practice, TEM is rarely used alone and is generally part of an integrated diagnostic protocol. The following criteria can be used to decide if TEM is appropriate (Stirling et al., 1999a, 2013a):

- When it provides useful structural, functional or compositional information.
- When only atypical or minor abnormalities are visible by light microscopy.
- When affinity labelling results are equivocal.
- When there is no realistic alternative or simple test available.
- For the investigation of new diseases and microorganisms.
- When it is time and cost effective in respect to alternative techniques.

Renal disease

The basic diagnostic features of the major renal diseases are outlined in Tables 21.4–21.9 and Figs. 21.7–21.20.

The location and morphology of immune complex deposits

Immune complex deposits are seen as accumulations of electron-dense, finely granular material in, or adjacent to, the glomerular basement membrane (GBM) and mesangial matrix (Figs. 21.7–21.12). Deposits may also have an 'organized' structure, most commonly fibrils (Figs. 21.13 and 21.14) or tubules (Herrera & Turbat-Herrera, 2010). The principal forms of deposit are (Stirling et al., 1999b):

- **Subepithelial (epimembranous):** raised dome-shaped deposits which protrude from the outer surface of the GBM, between the GBM and the visceral epithelial cell foot processes.

Large well-formed deposits, typical of post-infectious glomerulonephritis (GN), are termed 'humps' (Fig. 21.7). Smaller deposits are typical of membranous GN where the deposits may eventually encroach into the GBM, provoking a GBM reaction. In this case the GBM may become thickened, with 'spikes' of new membrane forming adjacent to the deposits. These deposits may eventually be completely surrounded by the GBM. Finally, where deposits have been absorbed, electron-lucent areas surrounded by thickened GBM may remain (Fig. 21.9).

- **Intramembranous:** nodular or, more rarely, linear deposits which are completely incorporated into the GBM such as in advanced membranous GN and membranoproliferative glomerulonephritis (MPGN) (mesangiocapillary GN). In C3 glomerulopathy (MPGN type ll) deposits may be linear or intermittent: in dense deposit disease (DDD) the deposit is a linear, uniform (non-granular) dense 'transformation' (Fig. 21.12) whilst in C3 glomerulonephritis (C3GN) deposits are multiple, ill-defined and less dense. In light-chain disease linear deposits are 'powdery'.
- **Subendothelial:** linear or plaque-like deposits situated between the inner (luminal) aspect of the GBM and the endothelium (Fig. 21.8). Subendothelial deposits may be massive and visible by LM. The latter are seen typically in systemic lupus erythematosus (SLE) as nodular hyaline 'thrombi' or 'wire-loop' capillary wall thickening (LM descriptors).
- **Mesangial:** deposits which lie completely within the mesangial matrix. Mesangial deposits may be massive and are seen typically in IgA disease (Fig. 21.10).
- **Paramesangial:** deposits within the peripheral mesangial matrix, particularly at the junction of the mesangium and loop GBM (Fig. 21.10).

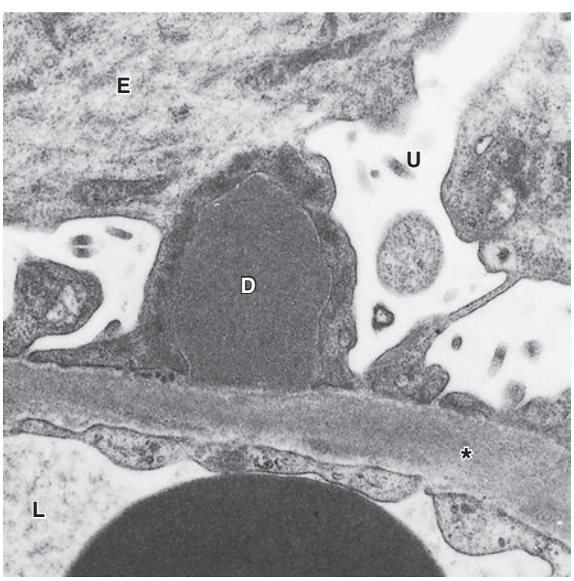


Fig. 21.7 Post-infectious GN. Typical subepithelial dome-shaped deposit 'hump' (D). GBM (*), epithelial cell cytoplasm (E), urinary space (U), capillary lumen (L) with part of a red blood cell also visible.

Variations in the thickness and/or texture of the GBM

The normal GBM has a mean thickness of approximately 390 nm within a range of 356–432 nm (Coleman et al., 1986) (Fig. 21.15).

The principal changes in the thickness and/or texture of the GBM are (Stirling et al., 1999b):

- **Thickness:** it may be thickened, thinned or irregular (Figs. 21.16–21.19).
- **Texture:** it may be laminated, fragmented or split. Electron-lucent zones may represent areas of resorbed deposits (Fig. 21.9).
- **Surface structure:** it may appear 'etched' (frayed or uneven).
- **Inclusions:** electron-dense granules and debris, microparticles, fibrils, fingerprint-like whorls, small vesicles and virus-like particles, fibrillar collagen and fibrin, all of doubtful or unknown diagnostic significance. Amyloid may also be found occasionally within the GBM (Fig. 21.13).
- **Folds and wrinkles:** folds and concertina-like wrinkling may result from ischemic collapse. Eventually, folds may consolidate as a thickened and laminated area of GBM; similar changes may also be seen around the periphery of the mesangium.

Table 21.4 Renal diseases with fine granular deposits: the ultrastructural features of post-infectious glomerulonephritis (GN), systemic lupus erythematosus (SLE), membranous GN, and IgA nephropathy

	Diagnostic ultrastructural features			
	Post-infectious GN	**Systemic lupus erythematosus (SLE)***	**Membranous GN**	**IgA nephropathy**
CAPILLARY WALL				
GBM morphology: contour, width, texture	Normal	Normal to irregular and thickened depending on the extent and location of membrane deposits.	**Stage I:** minimal irregular thickening. **Stage II:** marked thickening with membrane spikes (argyrophilic by LM). **Stage III:** thickened membrane surrounding deposits. **Stage IV and V:** much thickened with irregular patchy lucent areas common in stage V. (Stages II–V; Fig. 21.9)	Focal irregular thinning ('etching').
GBM deposit: type, location	Prominent subepithelial dome-shaped deposits (humps) are characteristic (Fig. 21.7): number of humps correlates approximately to intensity of inflammation. Intramembranous and subendothelial deposits may also be present.	Deposits increase in extent and location with severity of inflammation. GBM deposits (subepithelial, intramembranous, and subendothelial) (Fig. 21.8) indicate severe or global inflammation. In well-established disease, deposits may be found throughout the glomerulus. SLE can mimic other diseases because of the variety of damage caused.	**Stage I:** subepithelial deposits. **Stage II:** subepithelial deposits with membrane spikes. **Stage III:** intramembranous deposits. **Stage IV:** some deposits resorbed, leaving lucent areas. **Stage V:** many deposits resorbed, leaving poorly defined lucent and rarefied areas.	Subendothelial deposits variable but present in some cases. Subepithelial and intramembranous deposits rare.
Visceral epithelium	Foot processes usually effaced over humps so that their outer surface is covered by epithelial cell cytoplasm.	Cells may contain tubuloreticular inclusions.	Foot process effacement.	Focal foot process effacement.
Endothelium, subendothelial plane	Normal	Tubuloreticular inclusions common in endothelial cells (Fig. 21.20) (note that these may be found in small numbers in other renal diseases).	Normal	Normal

Continued

Table 21.4 Renal diseases with fine granular deposits: the ultrastructural features of post-infectious glomerulonephritis (GN), systemic lupus erythematosus (SLE), membranous GN, and IgA nephropathy—cont'd

	Diagnostic ultrastructural features			
	Post-infectious GN	**Systemic lupus erythematosus (SLE)***	**Membranous GN**	**IgA nephropathy**
MESANGIUM				
Matrix	Areas of matrix separated by cellular swelling, proliferation, and infiltration.	Diffuse expansion	Normal	Increased
Deposits	Peripheral humps with deposits common within matrix.	Deposits found in most classes.	Deposits absent in primary GN; may be present in secondary membranous GN.	Present, sometimes nodular (Fig. 21.10).
Cells	Proliferation (endocapillary) with infiltration by inflammatory cells including macrophages and polymorphs.	Diffuse but irregular proliferation with segmental inflammation.	No increase	Variable mesangial cell proliferation.

*Note: The features of SLE are variable and depend on the class of disease. See the ISN/RPS 2003 classification (Weening et al., 2004).

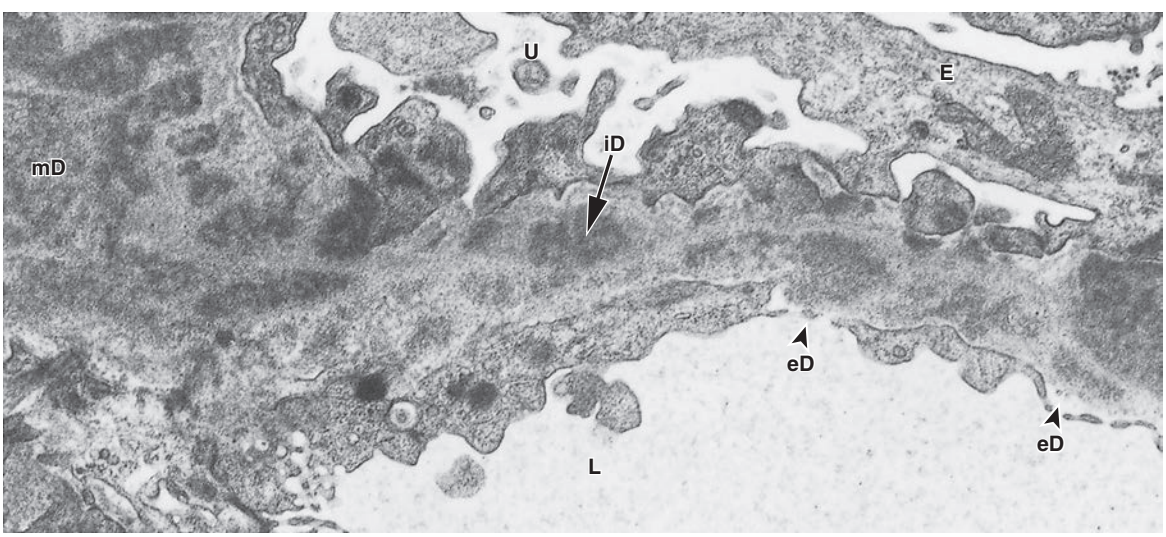


Fig. 21.8 Systemic lupus erythematosus. Subendothelial (eD) and intramembranous (iD) deposits are seen in the GBM; mesangial deposits (mD) are also present. Epithelial cell cytoplasm (E), urinary space (U), capillary lumen (L).

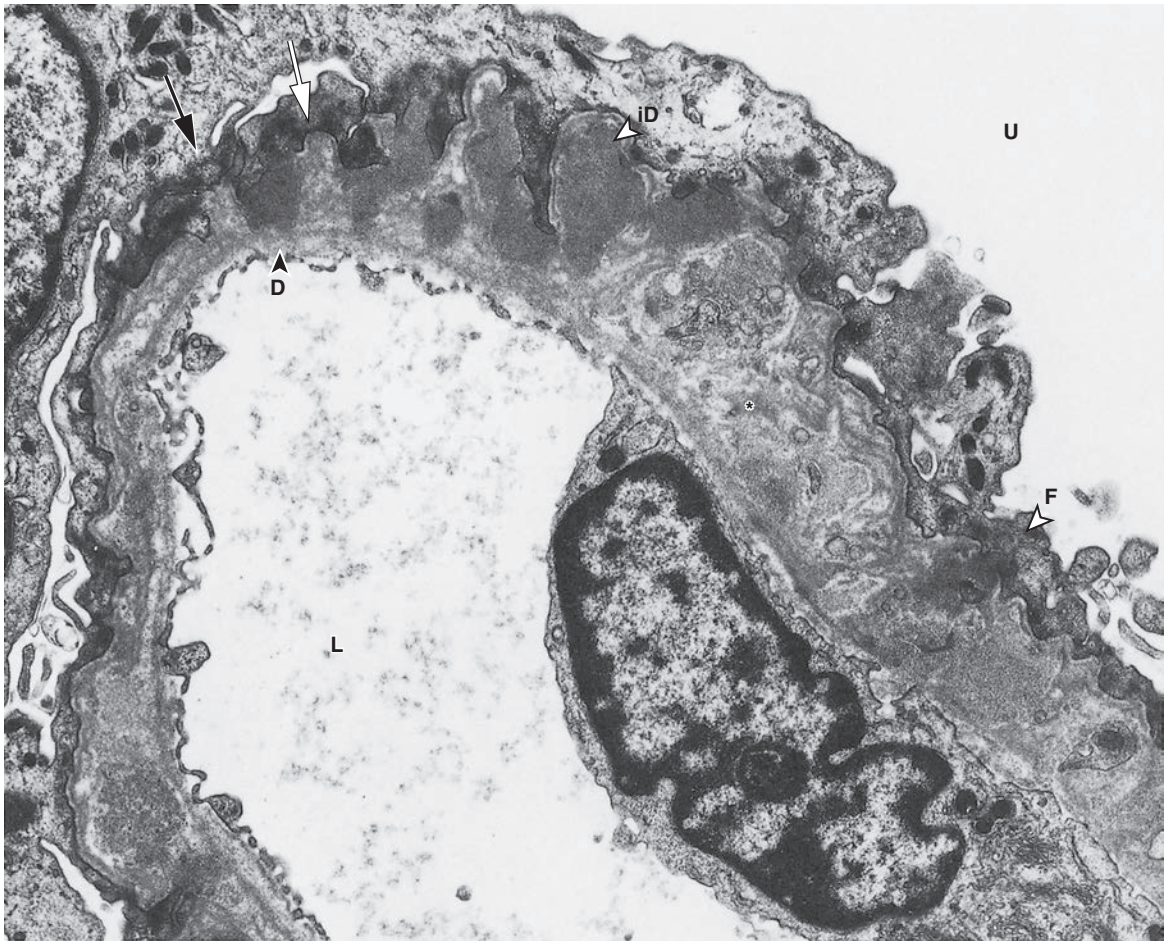


Fig. 21.9 Membranous GN Stages II–V. Stage II: subepithelial deposit (D) with membrane spikes (arrows) on both sides. Stage III: intramembranous deposit (iD). Stage IV–V: thickened and disrupted GBM with patches of partially resorbed deposit and lucent areas (*). Epithelial cell foot processes are extensively effaced (F). Urinary space (U), capillary lumen (L).

- **Double contouring (interposition):** the GBM appears duplicated due to the interposition of mesangial cells and matrix between the GBM and the endothelium in the capillary loop (sometimes called 'tram-tracking' when seen by LM) (Fig. 21.11).
- **Subendothelial widening:** the space between the endothelium and the GBM may become widened with an accumulation of flocculent material or, more rarely, cellular elements from the blood, e.g. in hemolytic uremic syndrome.
- **Gaps:** rarely, small discontinuities are seen in the GBM. Such gaps are of unknown diagnostic significance. Although it has been speculated that GBM discontinuities are responsible for hematuria, few are seen, even in cases of macroscopic hematuria.

Morphological and numerical changes in the cellular components of the glomerulus

Changes of diagnostic significance may also occur in the cellular elements of the glomerulus. The most significant include (Stirling et al., 1999b):

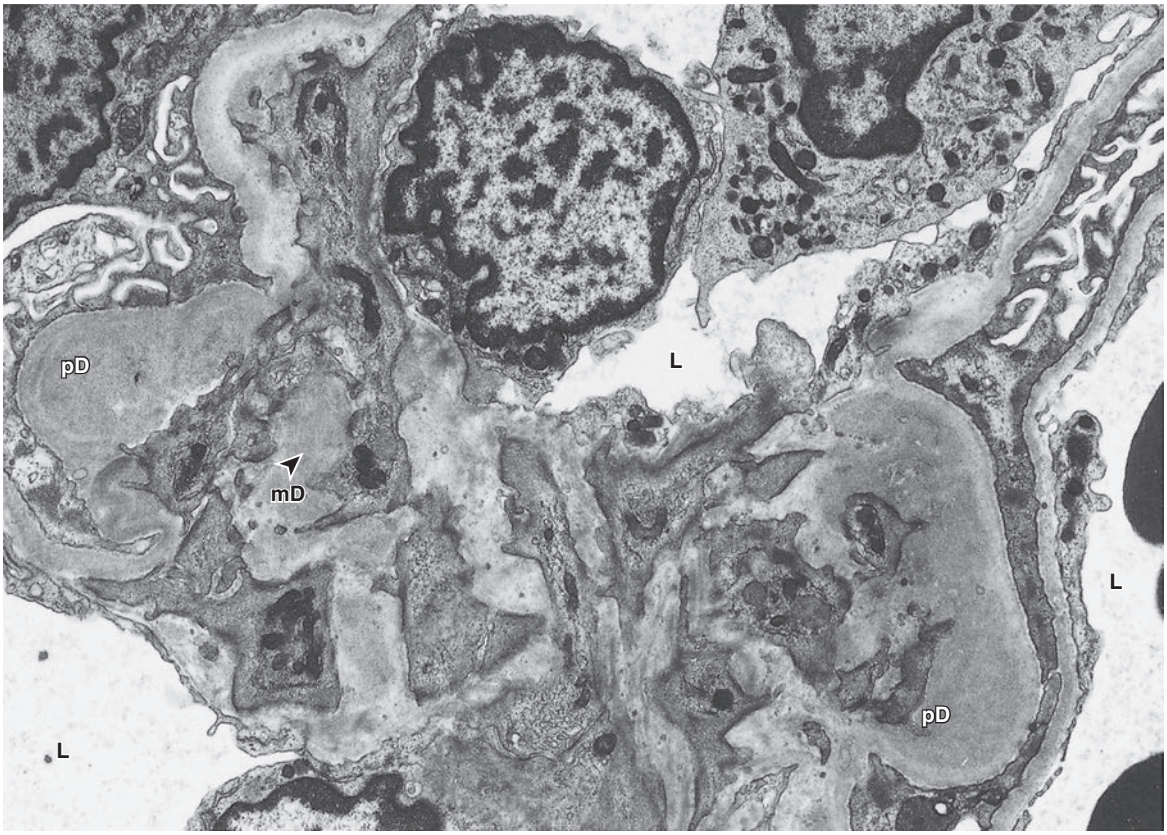


Fig. 21.10 IgA nephropathy. Areas of mesangial (mD) and nodular paramesangial (pD) deposit are seen within the mesangium. Capillary lumens (L).

Table 21.5 Renal diseases with fine granular deposits: the ultrastructural features of membranoproliferative glomerulonephritis (MPGN)

	Diagnostic ultrastructural features	
	MPGN type I (with subendothelial deposits)	**MPGN type II, C3 nephropathy: dense deposit disease***
CAPILLARY WALL		
GBM morphology: contour, width, texture	Double contouring ('tram tracking' by LM) due to mesangial interposition in well-developed disease (Fig. 21.11)	Interposition in some cases
GBM deposit: type, location	Deposits mainly in interposition zone (Fig. 21.11)	Linear dense deposit (transformation), often continuous (Fig. 21.12)
Visceral epithelium	Variable foot process effacement	Normal
Endothelium, subendothelial plane	Interposition of mesangial cells, with deposits and new GBM-like material (Fig. 21.11)	Mesangial interposition
MESANGIUM		
Matrix	Greatly increased	Increased
Deposits	Present	Present, dense and finely granular
Cells	Endocapillary proliferation	Endocapillary proliferation

*MPGN type II, C3 nephropathy: C3 glomerulonephritis (C3GN). Similar to DDD, has an MPGN morphology but with ill-defined non-linear electron dense deposits within the GBM and mesangium similar to those seen in immune complex GN with deposits in sub-endothelial or sub-epithelial locations (Cook, 2017). MPGN III has mesangial, subepithelial and subendothelial deposits (Chae, 2014).

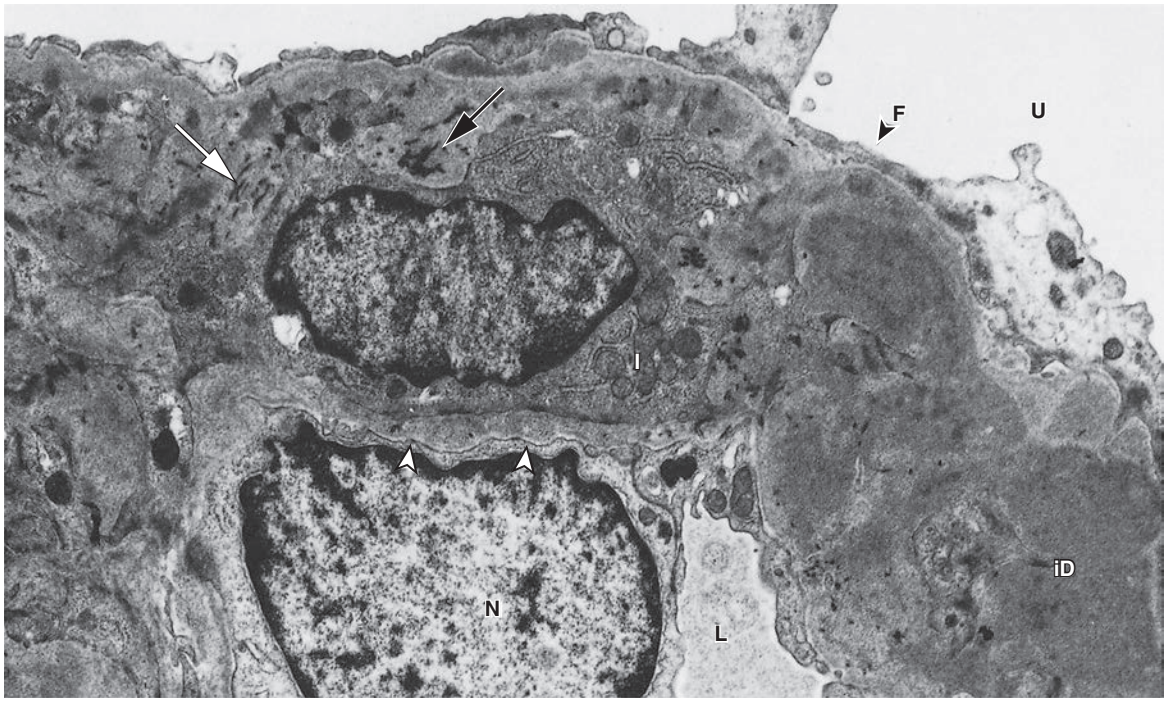


Fig. 21.11 MPGN type I. The capillary wall is considerably thickened due to mesangial cell interposition (I) and the formation of new basement membrane-like material (arrowheads) giving rise to the appearance of double contouring, as seen by LM. Collagen fibers (arrows) and large areas of intramembranous deposit (iD) can also be seen in the capillary wall. Epithelial cell foot processes are extensively effaced (F). Urinary space (U), capillary lumen (L), endothelial cell nucleus (N).

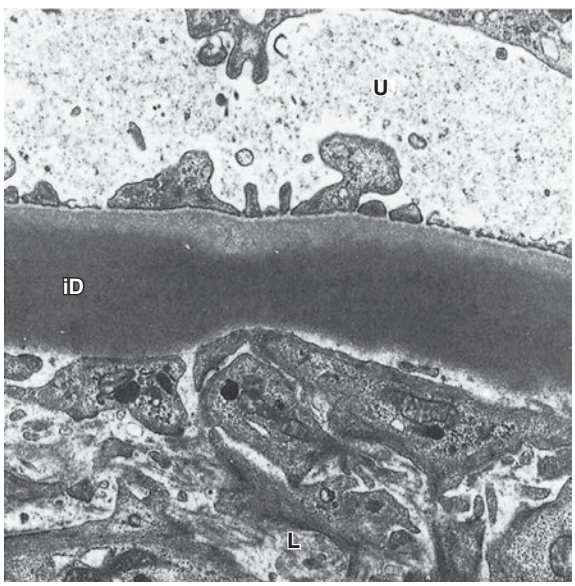


Fig. 21.12 MPGN type II, C3 nephropathy, dense deposit disease. A continuous linear zone of dense intramembranous deposit (iD) is seen along the length of the GBM. Cells of indeterminate type are seen in the capillary lumen (L). Urinary space (U).

- **Capillary endothelium:** cytoplasmic tubuloreticular inclusions may be found in the endothelial cytoplasm and are most common in SLE (Fig. 21.20).
- **Visceral epithelium:** the epithelial cell foot processes may be effaced to form a continuous (or semi-continuous) layer of cytoplasm (Fig. 21.17).
- **Mesangial cells:** mesangial cells may increase in number; the matrix may also be increased.

Renal transplants

TEM is used to evaluate renal transplant biopsies for transplant glomerulopathy, recurrent primary disease, de novo glomerular disease, infection (principally viruses and microsporidia) and when there is an inconclusive diagnosis by LM and/or immunofluorescence (Brealey, 2013). The features of recurrent and de novo glomerular disease are similar to those seen in native kidneys. The features of transplant glomerulopathy are shown in Table 21.9. Infections are generally due to immune suppression

Table 21.6 Renal diseases with fibrillar deposits*: the ultrastructural features of renal amyloid and immunotactoid glomerulopathy/fibrillary glomerulonephritis

	Diagnostic ultrastructural features	
	Amyloid	**Immunotactoid glomerulopathy/ fibrillary glomerulonephritis**
CAPILLARY WALL		
GBM morphology: contour, width, texture	Thickened and irregular due to deposition of amyloid fibrils.	Diffuse thickening frequent.
GBM deposit: type, location	Deposits Congo red positive by LM. Typical amyloid deposits: extracellular fine non-branching fibrils approximately 7–10 nm in diameter; variable in location. Fibrils tangled and irregular; not organized (Fig. 21.13).	Deposits Congo red negative by LM. Extracellular non-branching fibrils or tubules, variable in location, mostly randomly arranged but sometimes in parallel arrays. Fibrillary GN: solid fibrils approximately 13–29 nm in diameter (Fig. 21.14). Immunotactoid: tubules 10–90 nm in diameter, usually greater than 30 nm (Stirling and Curry, 2013)
Visceral epithelium	Often widespread foot process effacement.	Diffuse foot process effacement may be present.
Endothelium, subendothelial plane	Normal	Normal
MESANGIUM		
Matrix	Amyloid deposits.	Expansion may be present.
Deposits	Amyloid fibrils. Fibrils tangled and irregular; not organized (Fig. 21.13).	Mesangial deposits present in most cases. Fibrillar/tubular, mostly randomly arranged, 9–50 nm (or greater) in diameter.
Cells	Normal	Mild hypercellularity associated with deposits.

*Note, additional fibrils which may also found include cryoglobulins and a variety of nonimmune fibrils and filaments such as collagen and fibrin and the fibers found in Kimmelstiel-Wilson nodules in diabetic nephropathy (Stirling and Curry, 2013).

and co-infection of the graft with several organisms may occur.

Microsporidia found in the kidney (and urinary tract) include: *Enterocytozoon bieneusi*; *Encephalitozoon intestinalis, E. hellem and E. cuniculi*; and *Trachipleistophora anthropopthera* (Curry, 2013; Kicia et al., 2014). It is likely that additional species will also be found.

Viruses cause the majority of graft infections and are a significant cause of morbidity in transplant patients. Infection may be from an external source, including the transplant itself, or due to the reactivation of endogenous viruses. Common viruses found in renal transplants include: human polyomaviruses, especially JC and BK, the latter being the major type, are non-enveloped icosahedral DNA viruses, round to polygonal in shape and approximately 40-50 nm in diameter; herpes viruses are enveloped icosahedral DNA viruses, capsids are round to polygonal, approximately 100 nm in diameter and with a polygonal or rod-shaped core, enveloped viruses are approximately 120-150 nm adenoviruses are non-enveloped icosahedral DNA viruses, hexagonal in shape and

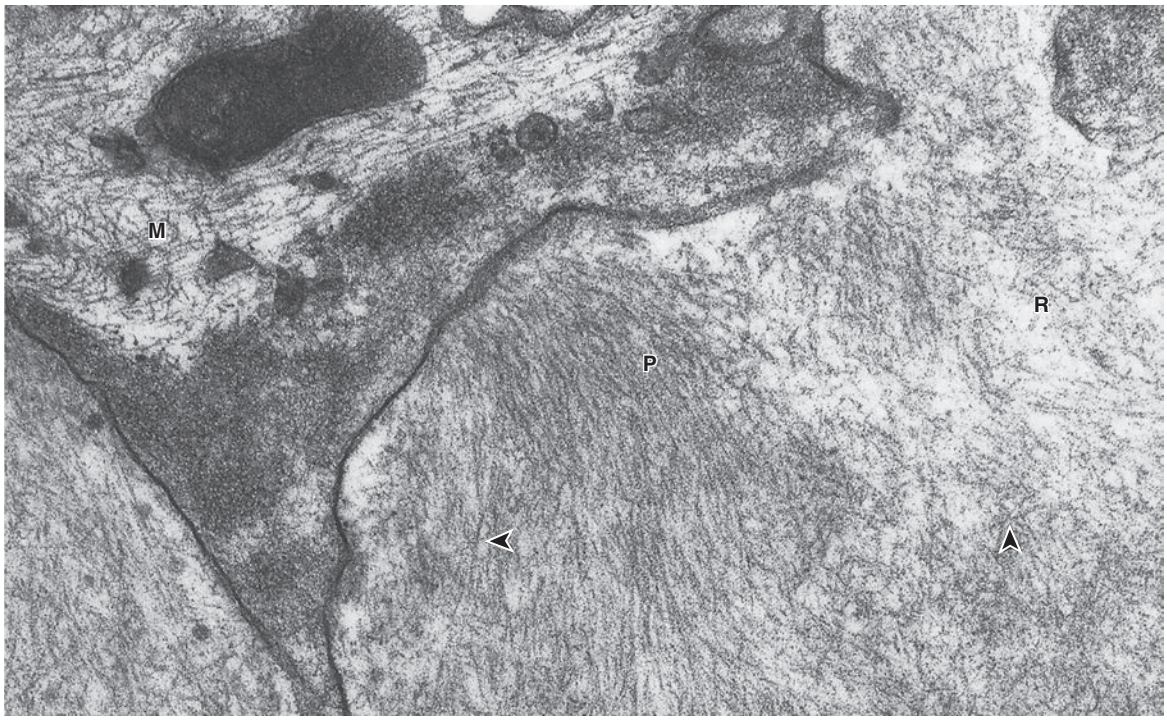


Fig. 21.13 Amyloid. Typical fine, non-branching, extracellular amyloid fibrils in the glomerular mesangium. The fibrils seen in this micrograph are arranged randomly (R) and in parallel (P). Individual fibrils (arrowheads) are 8–10 nm in diameter. Mesangial cell cytoplasm (M).

approximately 70-75 nm in diameter (Brealey, 2013). For a comprehensive review see Moal et al. (2013).

Malignant tumors

Mesothelioma

These tumors are morphologically diverse, with three major types generally recognized: epithelial, mixed (biphasic) and sarcomatoid (Ray & Kindler, 2009). Unusual variants and subtypes also occur and mesothelioma may mimic other tumor types (Henderson et al., 1992, 1997) making diagnosis problematic (Addis & Roche, 2009) and TEM is recommended when (Comin et al., 1997):

- The sample is small, e.g. cytological specimens, including cell block preparations.
- The histological appearances are atypical.
- The immunohistological findings are atypical.

For an unequivocal diagnosis of mesothelioma, mesothelial hyperplasia and metastatic tumor mimicking mesothelioma (especially adenocarcinoma) must be excluded (Henderson, 1982; Oury et al., 1998). Ultrastructural features which can help to distinguish between epithelial mesothelioma and adenocarcinoma include:

- **Microvilli:** mesothelial cells have longer microvilli than those of adenocarcinoma (Fig. 21.21) (Coleman et al., 1989; Henderson et al., 1992), with a mean length-to-diameter ratio (LDR) of 11.9 (standard deviation 5.87, range 4.8–21.3) (Warhol et al., 1982) versus a mean LDR of 5.28 (standard deviation 2.3, range 2.3–10).
- **Contact between stromal collagen fibrils and microvilli***:* in mesothelioma, microvilli may be found interdigitating, or in contact, with stromal

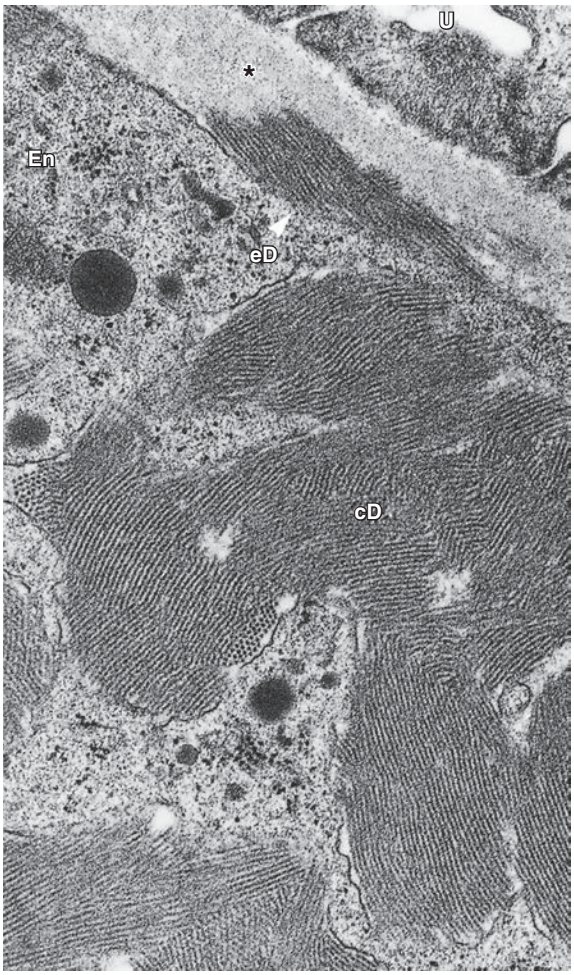


Fig. 21.14 Fibrillary GN. Fibrillar deposits are seen in the subendothelial zone (eD) and within the capillary lumen (cD). Individual fibrils are approximately 20 nm in diameter. GBM (*), endothelial cell cytoplasm (En), urinary space (U).

collagen fibrils (Fig. 21.21) (Carstens, 1992). This feature is also found occasionally in adenocarcinoma and is regarded as predictive of mesothelioma rather than an absolute discriminator (Carstens, 1992).

- **Cytoplasmic filaments:** intermediate filaments are common in mesothelioma where they are often aggregated into tonofilaments and are characteristically seen near the nucleus (Fig. 21.22) (Henderson et al., 1992).
- **Mucin granules:** these should be absent for a diagnosis of mesothelioma.

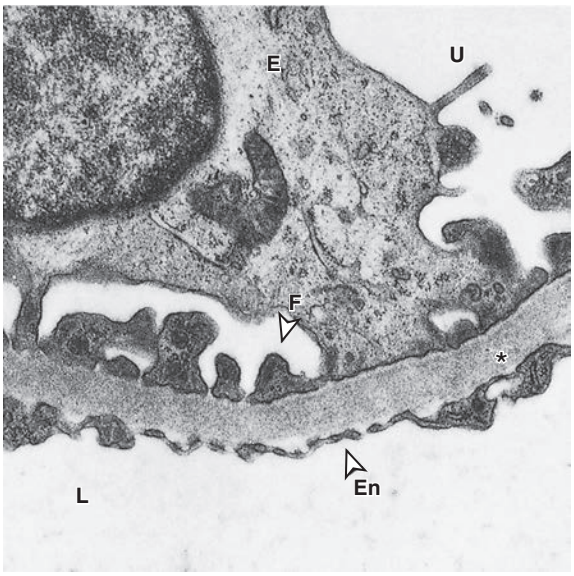


Fig. 21.15 Normal GBM. A length of normal GBM (*) with a mean width of approximately 390 nm. Compare this membrane with Figs. 21.16–21.19, which illustrate various types of abnormal GBM at the same magnification. Urinary space (U), epithelial cell (E) and foot processes (F), fenestrated endothelium (En), capillary lumen (L).

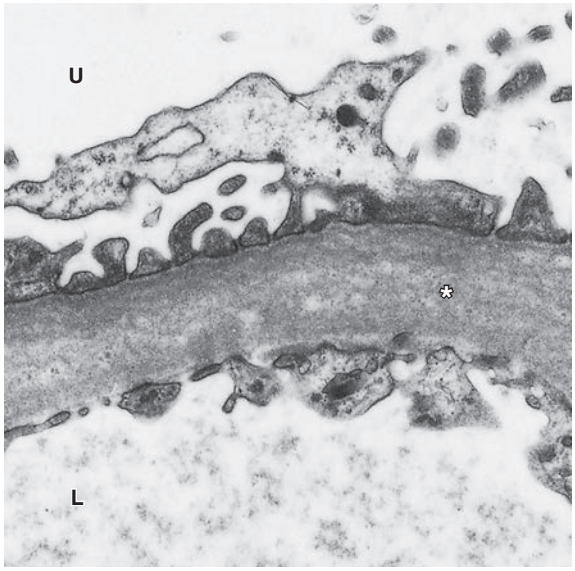


Fig. 21.16 Diabetes. In diabetes the GBM (*) typically shows uniform thickening. In this case the GBM is moderately thickened, with a mean width of approximately 919 nm. Urinary space (U), capillary lumen (L).

Table 21.7 Renal diseases with or without changes in GBM thickness: the ultrastructural features of diabetic glomerulosclerosis, minimal change disease, and nephrotic focal/segmental glomerulosclerosis

	Diagnostic ultrastructural features		
	Diabetic glomerulosclerosis	**Minimal change disease**	**Nephrotic focal/segmental glomerulosclerosis**
CAPILLARY WALL			
GBM morphology: contour, width, texture	Uniform increase in GBM thickness. Thickening may be considerable with GBM greater than 1000 nm in width (Fig. 21.16).	Variable thinning (Coleman & Stirling, 1991). Thinning minor but GBM may be less than approximately 300 nm in width (Fig. 21.17).	Segmental sclerosis; secondary ischemic change (GBM folding and consolidation).
GBM deposit: type, location	Nil	Nil	Nil
Visceral epithelium	Variable foot process effacement.	Diffuse foot process obliteration is the main feature (Fig. 21.17). Microvillous transformation.	Diffuse foot process effacement (segmental sclerosis and foot process effacement are essential for the diagnosis).
Endothelium, subendothelial plane	Normal	Normal	Normal
MESANGIUM			
Matrix	Increased, sometimes into nodular aggregates. Non-specific fibrils ~12 nm in diameter in Kimmelstiel-Wison nodules.	Normal	Segmental sclerosis in some cases, especially juxtamedullary glomeruli.
Deposits	Nil	Nil	Nil
Cells	Normal	Normal	Proliferation in some cases

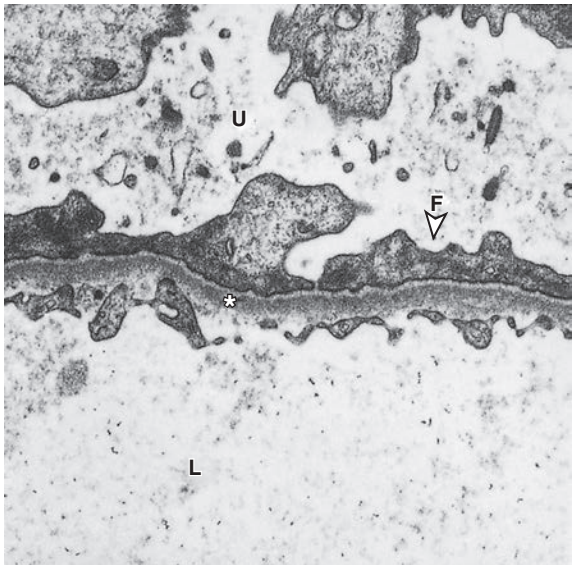


Fig. 21.17 Minimal change disease. The epithelial cell foot processes (F) are completely effaced. The GBM (*) is slightly thinned, with a mean width of approximately 226 nm. Urinary space (U), capillary lumen (L).

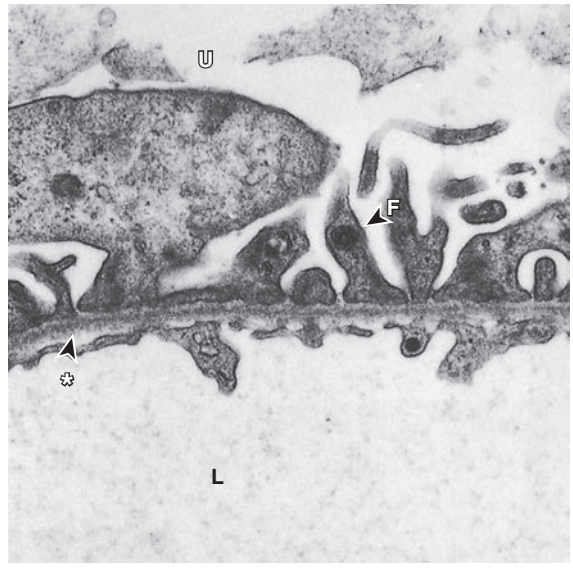


Fig. 21.18 Benign essential hematuria. The GBM (*) is extremely thin, with a mean width of approximately 183 nm. The foot processes (F) are generally intact but show minor areas of effacement ('smudging'). Urinary space (U), capillary lumen (L).

Table 21.8 Familial renal diseases with changes in GBM thickness or texture: the ultrastructural features of benign essential hematuria and Alport's syndrome

	Diagnostic ultrastructural features	
	Benign essential hematuria	**Alport's syndrome**
CAPILLARY WALL		
GBM morphology: contour, width, texture	Variable thinning is the main feature. Thinning may be considerable with GBM less than 150 nm in width (Fig. 21.18).	Alternating areas of thinning and thickening with lamellation (basket-weave pattern). Variability in width of GBM may be extreme (reported by Stirling et al., 1999b as 127–886 nm) (Fig. 21.19). Thickness calculations may be misleading, with overall GBM mean near normal value.
GBM deposit: type, location	Normal	Normal
Visceral epithelium	May show focal foot process effacement.	May show focal foot process effacement.
Endothelium, subendothelial plane	Normal	Normal
MESANGIUM		
Matrix	Normal	Normal
Deposits	Normal	Normal
Cells	Normal	Normal

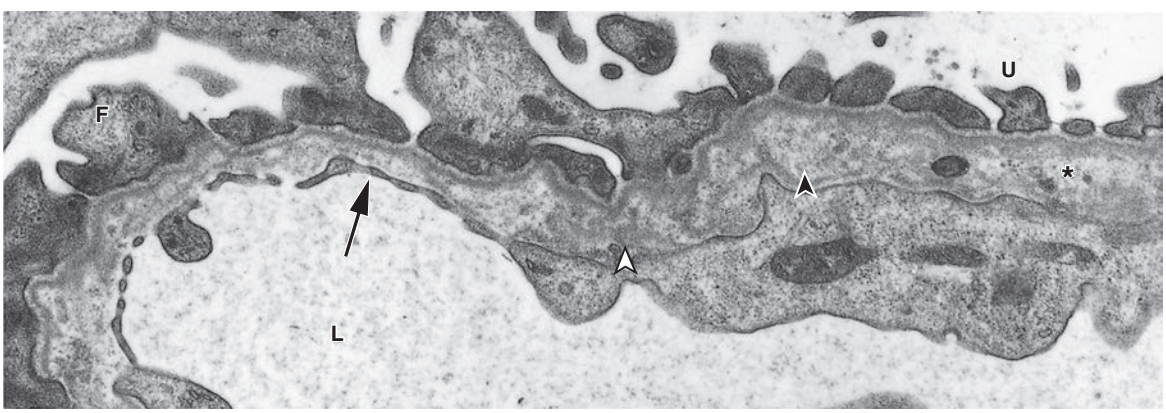


Fig. 21.19 Alport's syndrome. In Alport's syndrome the GBM may be extremely irregular, with thickened, thinned and lamellated areas. In this example the membrane is 260–900 nm in width. The thickened section of the GBM (*) is lamellated (arrowheads), foot processes are 'smudged' (F) and thinned area (arrow). Urinary space (U), capillary lumen (L).

Langerhans' histiocytosis (Histiocytosis X)

In Langerhans' histiocytosis (LH) the tumor cells are deviant macrophage-dendritic cells which are structurally similar to the Langerhans' histiocyte (Egeler et al., 2010). LH cells are identified at the ultrastructural level by the presence of Langerhans' cell granules (Birbeck or X bodies) (Fig. 21.23) which are induced by the expression of langerin (CD207) (Valladeau

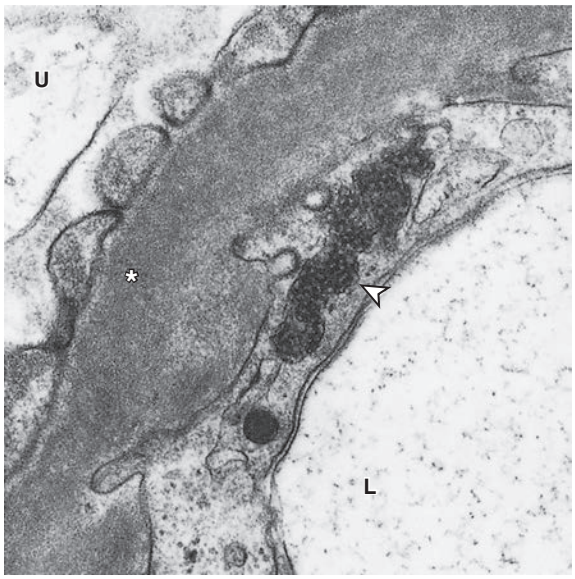


Fig. 21.20 Systemic lupus erythematosus. Tubuloreticular inclusion (arrowhead) in the cytoplasm of an endothelial cell. GBM (*), urinary space (U), capillary lumen (L).

et al., 2000). TEM has been used to diagnose LH for many years and remains useful, however, it should be noted that Birbeck bodies are not specific for LH as they may also be found in other disorders (Henderson et al., 1986; Erlandson, 1994). Immunohistochemical techniques are now regarded as superior to TEM, and characteristically the LCH immunophenotype includes CD1a, S100 protein, and langerin (CD207) with variable expression of CD68 (Harmon & Brown, 2015).

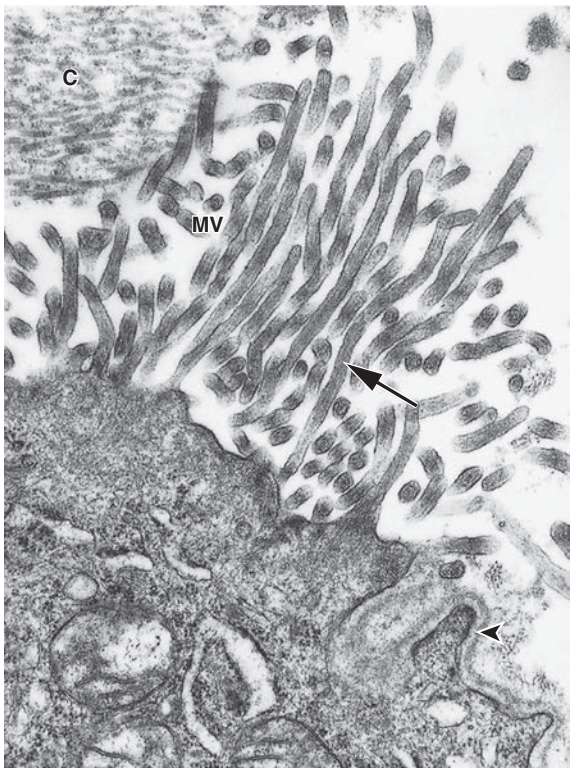


Fig. 21.21 Mesothelioma. The microvilli (MV) on the tumor cell shown here project through a discontinuous basal lamina (arrowhead) and are in contact with stromal collagen fibrils (C). In mesothelioma the microvilli are much longer than those of adenocarcinoma and have an LDR greater than 11.9. The microvillus marked (arrow) is approximately 1900 nm long and 86 nm wide (LDR = 22).

Non-neoplastic diseases

Skeletal muscle

A wide range of ultrastructural changes are seen in skeletal muscle in primary muscle diseases, as secondary events in neurological diseases, and in some systemic diseases. Since it is contractile, skeletal muscle is prone to sampling artifacts and must be biopsied and processed carefully. Practical guidelines are given by Pearl and Ghatak (1995) and Dubowitz et al. (2007). Only a small number of the ultrastructural changes which may be found are specific and diagnostically significant (Papadimitriou et al., 1992a; Stirling et al., 1999b); the principal features are summarized in Table 21.10.

Epidermolysis bullosa (mechanobullous dermatoses)

Epidermolysis bullosa (EB) is a heterogeneous group of rare, inherited or acquired diseases in which the skin blisters easily under normal levels of mechanical stress. Based on the level of blister formation within the dermal-epidermal junction, EB has traditionally been classified into three major groups: simplex, junctional and dystrophic (Anton-Lamprecht, 1992; Jaunzems & Woods,

Table 21.9 Renal transplant glomerulopathy – summary of major ultrastructural abnormalities (Brealey, 2013)

	Diagnostic ultrastructural features
Glomeruli	GBM reduplicated and thickened, with or without mesangial interposition. Reduplicated GBM in juxtamesangial area. Mesangia hypercellular and with expanded matrix. Translocation of endothelial cell nuclei from juxtamesangial area to the periphery of loops. Expanded areas of subendothelial lucency around GBM with flocculent or lace-like material. Endothelial cell hypertrophy and decreased fenestrations. May show finger-like protrusions into reduplicated GBM. Glomerulitis: numerous monocytes present in loops. Foot process effacement, may be severe in advanced disease (may not be distinguishable from focal segmental glomerulosclerosis).
Immune deposits	Lack of deposits in non-sclerotic areas.
Peritubular capillaries	Reduplication and/or splitting of basal lamina (transplant capillaropathy). Significant when reduplication is present in more than 60% of the vessel circumference and in the majority of vessels. Basal lamina changes are classified as: (I) normal, unilayered (50–70 nm); (II) thick unilayered (>200 nm); (III) mild splitting and reduplication (2–3 layers) and/or lace-like (up to 500 nm); (IV) moderate splitting and reduplication (4–6 layers) and/or lace-like (up to 500–1000 nm); (V) severe splitting and reduplication (>6 layers) and or lace-like (>1000 nm). Endothelial hypertrophy and decreased fenestrations.

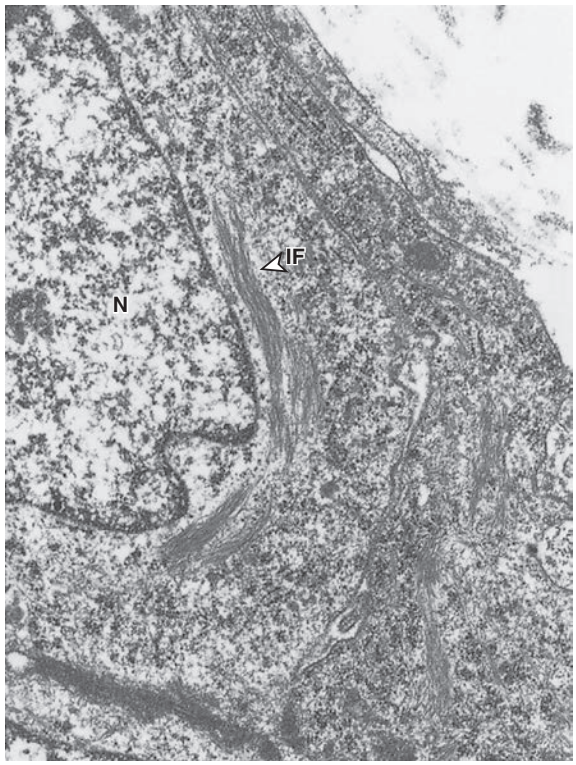


Fig. 21.22 Mesothelioma. Cytoplasmic intermediate filaments (IF) are common in mesothelioma, especially near the nucleus (N).

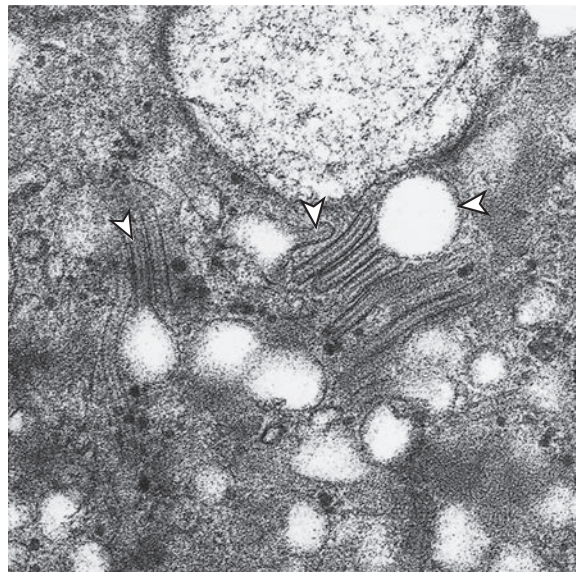


Fig. 21.23 Langerhans' cell granules (Birbeck bodies). Langerhans' cell granules (arrowheads) are typical of the Langerhans' histiocyte and the tumor cells of Langerhans' histiocytosis. The granules comprise a short rod-like structure with a clear vesicle at one end.

Table 21.10 Skeletal muscle – summary of major ultrastructural abnormalities (Schochet & Lampert, 1978; Papadimitriou et al., 1992a; Stirling et al., 1999b)

Structural element	Alteration and disease state
Satellite cells	Rare in normal muscle. Frequent in regenerating and denervated muscle. Increased in polymyositis, Duchenne muscular dystrophy, congenital myotonic dystrophy, Werdnig-Hoffman disease, and Kugelberg-Welander syndrome. May display evidence of activation and myogenic differentiation. May be confused with invading inflammatory cells.
Nuclei	Large internal nuclei in recently regenerated myofibers. Contours convoluted in atrophy and nemaline myopathy. Abundant internal nuclei found in various myopathies. Internal nuclei especially numerous, and arranged in chains, in myotonic dystrophy. Chains of internal nuclei a distinctive feature of centronuclear (myotubular) myopathy. A variety of vacuoles, inclusions, and pseudoinclusions present in a wide range of diseases. Nemaline bodies found in a few cases of polymyositis and some cases of late-onset rod diseases (see Z-discs below). Filamentous intranuclear inclusions resembling myxovirus in polymyositis and chronic distal myopathy. Fibrillar inclusions present in inclusion body myositis and polymyositis. Annulate lamellae present in a range of diseases.
Myofibrils	Hypercontracted myofibrils non-specific and often artifactual. Aberrant bundles of normal fibrils spiraling, or encircling, the long axis of myofibers are frequent in myotonic dystrophy. This feature is also observed in other diseases. Sarcomeres with disorganized myofibrils are non-specific but common in congenital myopathies (multicore and minicore diseases). Extensive disorganization of central region of type 1 myofibers is the major lesion in 'target' and 'core-targetoid' fibers. Target fibers occur in denervation, re-innervation, polymyositis and familial periodic paralysis. Core/targetoid lesions present in denervating and myopathic conditions and in the aged. Peripheral subsarcolemmal aggregates of disorganized myofibrils and sarcoplasm are found in a variety of disorders but are characteristic of myotonic dystrophy.
Z-discs	Z-disc abnormalities are common in many disease states. Streaming of Z-disc material, Z-disc duplication, and zig-zag irregularities are the most common lesions. Characteristic rod-shaped electron-dense bodies (nemaline bodies), 6–7 μm long and similar in appearance to Z-discs, are common in nemaline myopathy. These bodies contain actin and α-actinin and are also found sporadically in other diseases. Widespread loss of Z-disc material noted in a variety of diseases. Discrete osmiophilic cytoplasmic bodies, thought to be related to Z-discs, noted in a wide range of diseased myofibers.
Mitochondria	Swelling, with deposition of osmiophilic material or formation of myelin figures, common and non-specific. Swelling may result from suboptimal fixation. Changes in numbers common and non-specific. Re-orientation of intermyofibrillar mitochondria (in relation to myofiber) occurs in a range of diseases. Structural abnormalities common (some associated with biochemical deficiencies) and present in a wide range of diseases, including the 'mitochondrial myopathies' and 'mitochondrial encephalomyopathies'. Electron-dense granules and crystalline inclusions present in a wide range of diseases.

Continued

Table 21.10 Skeletal muscle – summary of major ultrastructural abnormalities (Schochet & Lampert, 1978; Papadimitriou et al., 1992a; Stirling et al., 1999b)—cont'd

Structural element	Alteration and disease state
Transverse tubular system	Abnormalities in triads common in injured and atrophic fibers. Dilation is a common artifact but may also be present in a variety of diseases. Coalescence of T-system tubules to give a honeycomb pattern may be present in a wide range of diseases.
Sarcoplasmic reticulum	Dilation of cisternae prominent in periodic paralyses and some other diseases. Elongated tubular aggregates (probably derived from sarcoplasmic reticulum) reported in the periodic paralyses and other diseases. Cylindrical structures in a spiral pattern, and with a core of glycogen, observed in a variety of diseases.
Inclusions and deposits	Filamentous bodies, concentric laminated bodies, zebra-striped bodies, fingerprint bodies, reducing bodies, spheroidal bodies, and paracrystalline arrays present in the sarcoplasm in a range of diseases. Excessive lipid accumulation present in a wide range of diseases. Glycogen abundant in fetal muscle and regenerating myofibers. Glycogen moderately increased in a range of diseases; massively increased in various glycogenoses. Autophagic vacuoles and lipopigments present in degenerative diseases and in almost any myopathic state.

1997; Jaunzems et al., 1997). More recently, a new classification system for inherited types of EB, the 'onion skin' approach, has been proposed. This system includes (Fine et al., 2014):

- The major EB type present, based on skin cleavage level.
- The phenotypic characteristics present.
- The mode of inheritance.
- The targeted protein and its relative expression in skin.
- The gene involved and type(s) of mutation present.
- If possible, the specific mutation(s) and their location(s).

Correspondingly, laboratory diagnosis is a multipronged strategy which includes TEM, immunofluorescence and mutation analysis (Fine et al., 2014; Intong & Murrell, 2012).

TEM allows the precise level of blister formation to be determined in all types of EB, as well as a detailed ultrastructural assessment of the basement membrane components. For a detailed description of the ultrastructural and immunohistochemical features of inherited EB see Fine et al. (2008). Acquired EB is an autoimmune disease which targets type VII collagen and is mainly diagnosed using histology, immunofluorescence, immunoblotting, and ELISA. Immunoelectron microscopy may also be useful (Kim & Kim, 2013).

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)

CADASIL is a familial form of early-onset vascular dementia associated with mutations in chromosome 19 (Notch 3 gene); at least 230 mutations in exons 2-24 have been reported (Tikka et al., 2014). In affected vessels, basophilic, periodic acid-Schiff positive deposits accumulate between the smooth muscle cells of the vessel walls. At the ultrastructural level these classic CADASIL-type deposits are often referred to as granular osmiophilic material (GOM) (Tikka et al., 2014).

Genetic screening for such a wide range of mutations is difficult. Strategies for diagnosis will depend on the patient's background and the presence of known mutations which will allow genetic testing to be targeted to a limited number of exons. Although the sensitivity of TEM screening of skin biopsies has

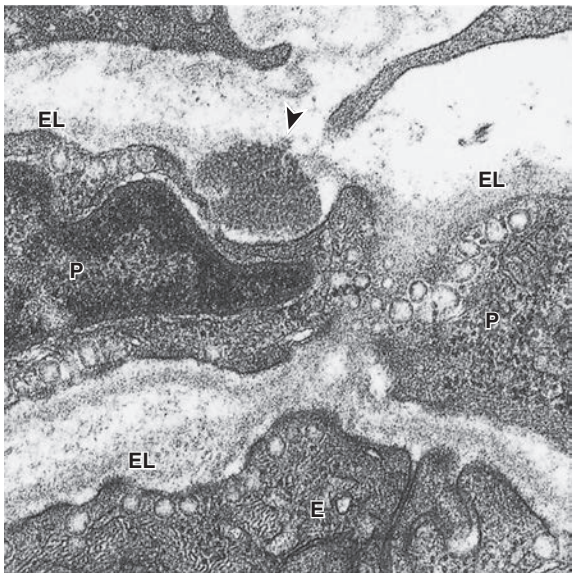


Fig. 21.24 CADASIL. In affected vessels electron-dense material (arrowhead) is seen in close proximity to the pericytes or perivascular smooth muscle cells. The material is often sited (as seen here) in an indentation in the cell wall. Pericytes (P), external lamina (EL), capillary endothelial cell (E).

been debated, a retrospective study of genetically positive CADASIL cases by Tikka et al. (2009) found typical GOMs in all the patients tested. For TEM screening, a 4 mm punch biopsy of skin is generally adequate. However, the tissue must be well fixed in glutaraldehyde as tissue fixed in formalin may not be adequate due to poor quality fixation and the presence of confusing non-specific electron-dense material. GOMs may be patchy in distribution so, to ensure that enough tissue is screened, it is recommended that a minimum of three blocks and 50 vessels are observed. Furthermore, as GOMs are more common adjacent to larger vessels, Tikka et al. (2009) also recommend that the material should include a number of small arterial vessels with well-differentiated pericytes or smooth muscle cells such as those found in the border zone between deep dermis and upper subcutis.

Ultrastructurally, GOMs are seen as extracellular electron-dense granular material which is often in contact with the vascular pericytes and smooth muscle cells, and sited in a small indentation (Fig. 21.24). Both GOMs and non-specific granular osmiophilic material may also be found within the intercellular stroma, but for an unequivocal diagnosis, GOMs sited in cellular indentations must be identified (Tikka et al., 2009, 2014).

Amyloid

Amyloid deposition is associated with a wide range of disorders and can either be hereditary or acquired. Amyloid deposits may also be focal, localized or systemic (Gillmore et al., 1997). Using TEM, amyloid is seen as randomly arranged extracellular non-branching fibrils. Individual fibrils are of indeterminate length and approximately 7–10 nm in diameter (Fig. 21.13) (Harvey & Anton-Lamprecht, 1992; Gillmore et al., 1997). See Chapter 15.

Cornea

TEM is useful for identifying corneal deposits and inclusions, particularly amyloid and immunoglobulin deposits in diseases such as paraproteinemic crystalloidal keratopathy (PCK). In PCK, immunoglobulin deposits may be seen in the corneal stroma either as organized or randomly arranged extracellular tubules (Fig. 21.25) (Stirling & Graff, 1995; Stirling et al., 1997), or as intracellular crystalloids with a fine fibrillar substructure (Fig. 21.26) (Henderson et al., 1993).

Cilia

Cilia are small motile structures approximately 5–10 μm long and 0.5 μm in diameter. Within the ciliary shaft there is a core of microtubules (the axoneme) composed of nine outer pairs of microtubules and one inner (central) pair, an arrangement referred to as the '9 + 2' configuration (Fig. 21.27) (Yiallouros et al., 2013).

A wide range of primary and secondary structural defects may be found in cilia; secondary defects, such as disorganized microtubules, can be ignored (Sturgess & Turner, 1984; Carson et al., 1994). Primary defects are caused by a heterogeneous group of genetic abnormalities which result in immotile cilia syndrome, primary ciliary dyskinesia (PCD) and a range of associated diseases

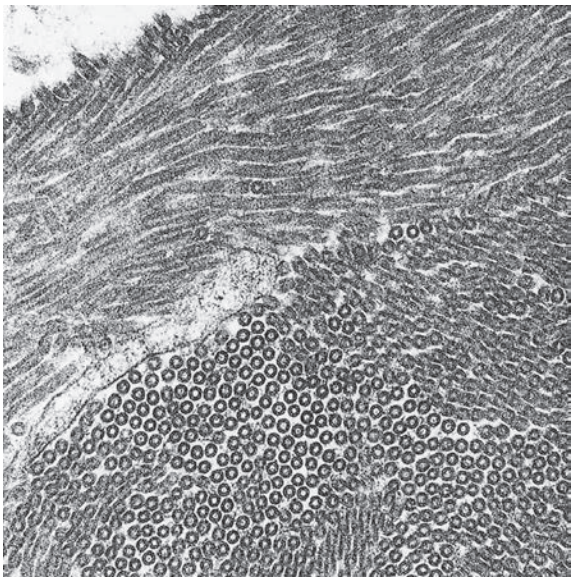


Fig. 21.25 Paraproteinemic crystalloidal keratopathy. Extracellular tubular crystalloids are found throughout the corneal stroma in this case. Tubules are thick-walled and of indeterminate length. Overall tubule diameter is 40–45 nm. The crystalloids labeled for κ-light chains in immunogold labeling studies (Henderson et al., 1993).

(Yiallouros et al., 2013, Meeks & Bush, 2000). In PCD, the ciliary defects are permanent and all cilia in the body are affected (Corrin & Dewar, 1992; Mierau et al., 1992). With this in mind it is generally said that the finding of a single normal cilium mitigates against a diagnosis of PCD. However, it should also be noted that not all genetic cilia defects result in abnormal ciliary morphology (Santamaria et al., 1999).

In general, it is recommended that screening for PCD should combine a clinical work up with an analysis of cilial beat frequency and pattern using high-resolution digital high-speed video (DHSV), immunofluorescence microscopy, nasal nitric oxide measurements, molecular genetics and an ultrastructural analysis, TEM being essential for accurate diagnosis (Yiallouros et al., 2013). The principal morphological defects are (Sturgess & Turner, 1984; Corrin & Dewar, 1992; Mierau et al., 1992; Carson et al., 1994):

- Outer and/or inner dynein arms absent or short (most cases) (Fig. 21.28).
- Ciliary spokes absent.

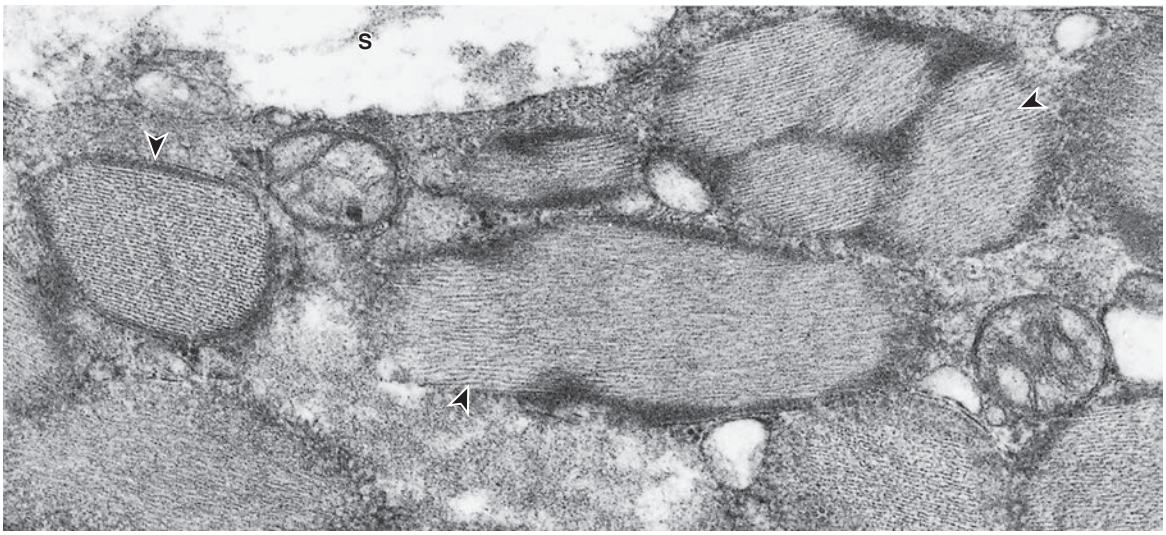


Fig. 21.26 Paraproteinemic crystalloidal keratopathy. Intracellular fibrillary crystalloids (arrowheads) in the cytoplasm of a corneal keratinocyte. Filaments are approximately 8–10 nm in diameter. The crystalloids labeled for κ-light chains in immunogold labeling studies (Henderson et al., 1993). Extracellular stroma (S).

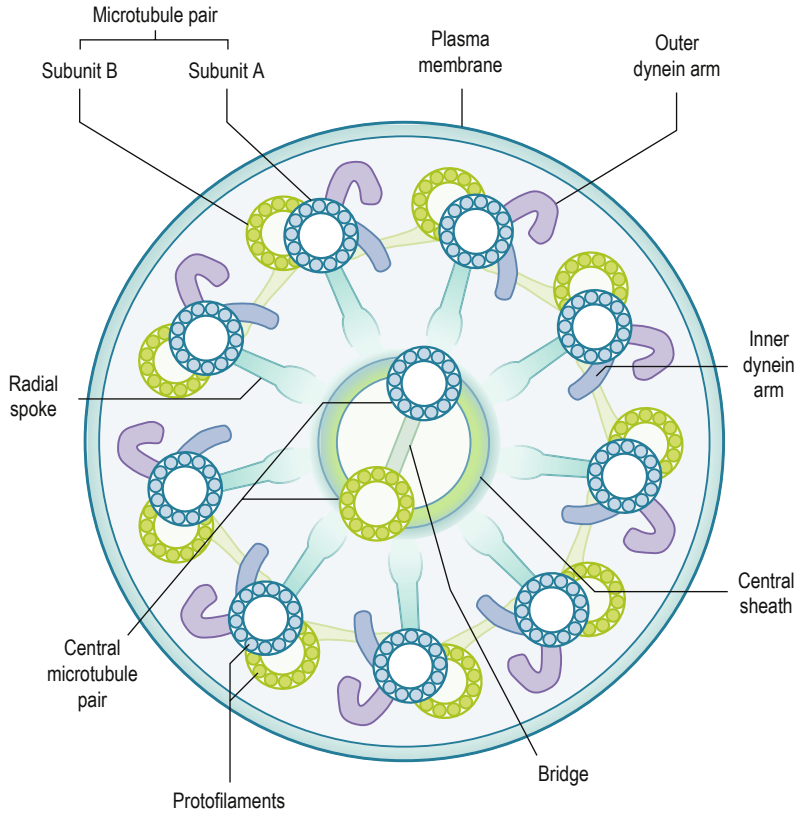


Fig. 21.27 Normal cilium. Schematic cross-section through the middle of a normal ciliary shaft to show the structure of the axoneme. The axoneme is formed from an outer ring of nine microtubule pairs with one central pair (the 9 + 2 configuration). The outer microtubule pairs are formed from two subunits (A and B); each subunit is formed from a ring of protofilaments. Projecting from each complete microtubule in the outer microtubule pairs (subunit A) is a pair of inner and outer dynein arms. A variety of structures (the radial spokes, bridge and sheath) appear to link the tubule pairs together.

- Outer microtubular pairs absent, displaced, or discontinuous.
- Central microtubular pair with one or both microtubules absent.

Observations should be made in the middle of the ciliary shaft, and approximately 50 cilia are recommended as a minimum number for examination. In routinely processed specimens the inner arms are often indistinct. Whilst this is undoubtedly a processing artifact, and such cilia are generally presumed to be normal, care must be taken to identify genuine inner arm defects.

Microsporidia

These are a group of obligate intracellular parasites belonging to the phylum Microspora (Curry, 1998; Wasson & Peper, 2000; Smith, 2009). Unclassified organisms are conveniently called by the collective name 'microsporidium' but this is not an official genus. *Enterocytozoon* is the most common species infecting humans (Fig. 21.29) but an increasing number of species are being identified (Table 21.11) (Didier et al., 2009). In some cases the identification of the organism involved is preliminary or tentative. Microsporidia may be found in almost any human tissue and, whilst the majority

Table 21.11 Microsporidia found in humans

Species	Citation
*Anncaliia algerae** *(Nosema algerae)*	Visvesvara et al., 1999
*Anncaliia connori** *(Nosema connori)*	Sprague, 1974; Cali et al., 1998
*Anncaliia vesicularum**	Cali et al., 1998
Encephalitozoon cuniculi	Pakes et al., 1975; Canning et al., 1986
Encephalitozoon hellem (Fig. 21.30)	Didier et al., 1991a, 1991b
Encephalitozoon (Septata) intestinalis	Cali et al., 1993; Hartskeerl et al., 1995
Endoreticulatus-like species	Pariyakanok et al., 2015
Enterocytozoon bieneusi (Fig. 21.29)	Desportes et al., 1985; Curry, 2000
Microsporidium africanum#	Pinnolis et al., 1981
Microsporidium ceylonensis#	Ashton & Wirasinha, 1973; Canning et al., 1998
Nosema sp.	Curry et al., 2007
Nosema ocularum	Cali et al., 1991
Pleistophora sp.	Canning et al., 1986; Weber et al., 1994
Pleistophora ronneafiei	Cali & Takvorian, 2003; Cali et al., 2005
Trachipleistophora anthropophthera	Vavra et al., 1998
Trachipleistophora hominis	Hollister et al., 1996
Tubulinosema sp.	Choudhary et al., 2011
Vittaforma corneae (Nosema corneum)	Silveira & Canning, 1995
Vittaforma-like species	Sulaiman et al., 2003

* *Anncaliia* replaces the genus *Brachiola* (Franzen et al., 2006).
# Unclassified organisms are conveniently called by the collective name 'microsporidium': this is not an official genus.

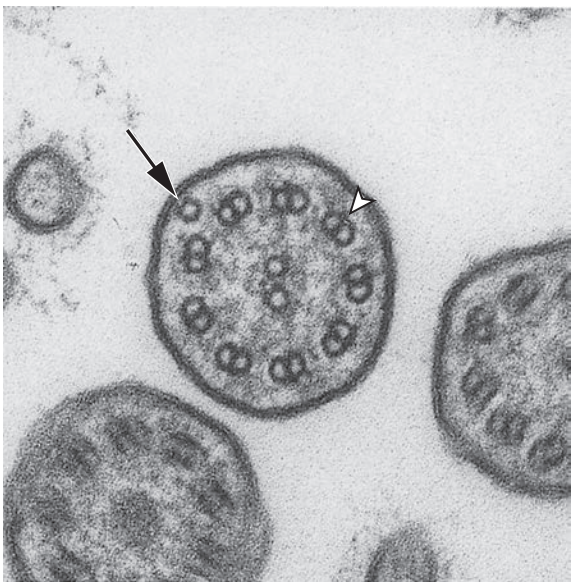


Fig. 21.28 Primary ciliary dyskinesia: dynein arm defect. The outer microtubule pairs (arrowhead) lack dynein arms. A single extra displaced microtubule is also present (arrow).

of infections occur in immunocompromised individuals, healthy people may also be infected. The eye is particularly susceptible, especially if the use of topical steroids creates a locally immunocompromised state (Chan et al., 2003; Badenoch et al., 2011). Co-infection by two species may also occur (Didier et al., 2009).

Light microscopy is the primary technique for confirming the presence of microsporidia which are visible using a wide range of stains (Ramanan & Prit, 2014). However, TEM plays an important role in identification and speciation and is regarded by some as the gold standard for diagnosis (Curry, 2000). The organisms can be identified in fecal material or tissue biopsies using standard TEM fixation and processing protocols (Weber et al., 1994). Miller and Simakova (2009) have suggested that staining with oolong tea extract may enhance the visibility of the

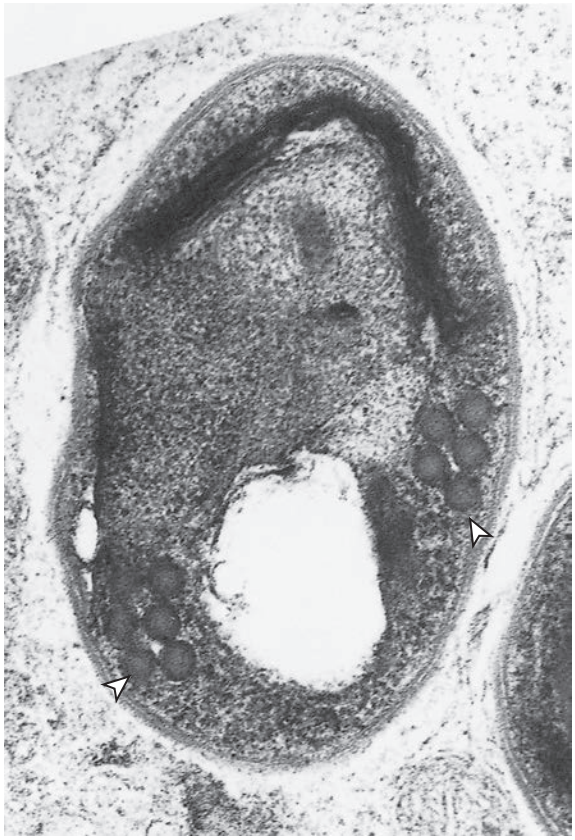


Fig. 21.29 *Enterocytozoon bieneusi*. Maturing spore showing the coils of the polar tube. In *E. bieneusi* the polar tube has a range of 4–7 coils which, in cross-section, are seen arranged in two rows on either side of the spore. The spore shown has 5–6 coils on either side (arrowheads). (Electron micrograph courtesy of Dr Alan Curry.)

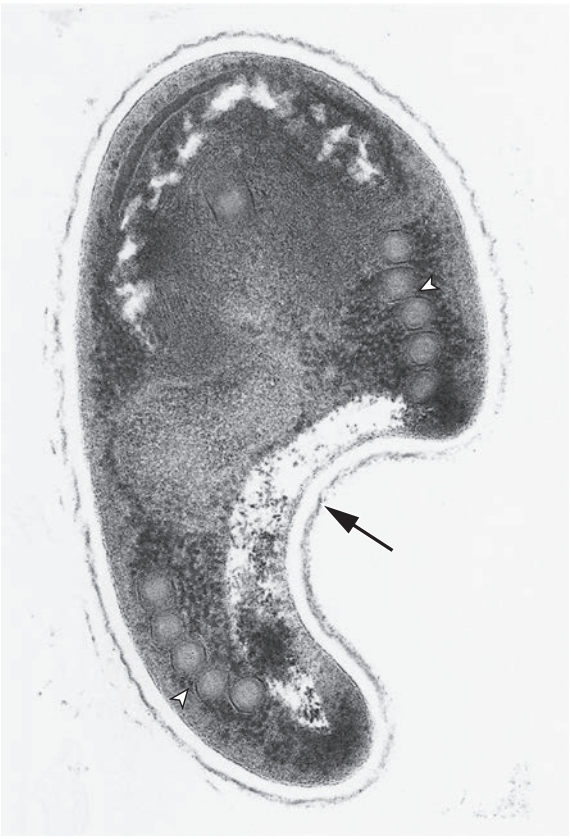


Fig. 21.30 *Encephalitozoon hellem*. Spore of *E. hellem* showing the polar tube with five coils arranged in a single row on each side of the spore (arrowheads). The indentation (arrow) is an artifact caused by the collapse of the posterior vacuole. (Electron micrograph courtesy of Dr Alan Curry).

substructure of the polar filament (Sato et al., 2008; Yamaguchi et al., 2010).

The major ultrastructural features used for typing microsporidia (Weber et al., 1994; Curry, 1998; Ramanan & Prit, 2014) are as follows:

- The size and morphology of the various developmental stages.
- The configuration of the nuclei in spores and developmental stages.
- The host-parasite interface.
- The number, arrangement and diameter of coils in the tubular extrusion apparatus (polar tube) in spores (Figs 21.29 and 21.30).

Acknowledgments

We thank Richard Davey (SA Pathology) for his assistance in preparing the illustrations for this chapter and Dr Alan Curry (Manchester Royal Infirmary) for his helpful advice on microsporidia.

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22 Digital pathology

Jonathan Bury • Jonathan Griffin

Key points

Whole slide imaging (WSI) allows the creation of digital images of entire histology and cytology slides with sufficient detail to allow viewing at varying magnifications comparable to that achievable with a conventional light microscope.

Acquiring digital WSIs requires dedicated hardware systems which create composite images of individually acquired tiles or lines. Such slide scanners typically include slide loading mechanisms, a motorized stage, a light path, image capture device and software to create the composite image. Although the data files which represent these images are large, software techniques enable these images to be viewed on screen in a manner analogous to how a slide may be viewed on a physical microscope.

Digital representation of slides enables histology images to be distributed, viewed and shared over computer networks rather than relying on physical handling of the glass slide. This is likely to represent the future of histology and cytology departments.

Introduction

Whole-slide imaging refers to the creation of a digital representation of the image presented by a glass histology slide, at a level of detail comparable to that seen with a light microscope. 'Digital pathology' is a broader term encompassing related processes which maximize the practical utility of such images, including the storage, viewing, annotation and use in applications including educational, research and clinical practice.

Benefits of digital images over physical glass slides

Digital images have several advantages over glass slides. Unlike a physical object, a digital image file can be moved from one physical location to another almost instantaneously. Indeed, an image file can be viewed at the same time by two, or more pathologists, in different locations and potentially separated by thousands of miles. With appropriate data-security arrangements, digital images should be more durable than glass slides which are physical, fragile and prone to fading. The overhead of sorting, filing, storing and retrieving glass slides is particularly burdensome on larger laboratories and a fully digital workflow has the potential to significantly reduce this. Finally, digital images are a prerequisite for automated image analysis.

Digital images

Images can be represented in numerical form in a variety of manners. The text on this page, for example, is ultimately represented by the printing software, not as letters and words but as collections of lines and curves. The commonest method for representing complex real world scenes, including histology images, is to consider the image as a grid of individual points, each with brightness and, for color images, hue. These individual points are referred to as pixels, the term pixel being a contraction of 'picture element' and the smallest resolvable detail. In all commonly encountered digital image formats pixels are square, although other shapes, in particular hexagons, are in principle possible. This approach represents images

mathematically as a matrix of brightness and hue values. The perceived quality of a digital image of this type relates to the total number of pixels (resolution) and a parameter called pixel depth.

Resolution

The size of the smallest resolvable detail in a whole slide image is defined by the original absolute size of the area represented by each pixel. This is determined by the quality of the slide scanner optics and sensor. Although WSI system vendors often refer to image quality as 'x40 equivalent' or 'x20 equivalent', reference to microns/pixel is preferable since this is an unambiguous measure of image resolution, unaffected by downstream variables such as monitor resolution and viewing distance (Sellaro et al., 2013). Broadly however, when viewed under appropriate conditions, images in which each pixel represents a square of side 0.5 microns (0.5 µm/pixel) are regarded as providing an equivalent level of detail to that seen with a x20 objective on a high quality microscope, whilst 0.25 µm/pixel is regarded as comparable to a x40 objective.

Pixel depth

Image quality is not defined solely by resolution. The term 'pixel depth' refers to the extent to which subtly different colors can be distinguished. The crude but recognizable image in Fig. 22.1 has a pixel depth of 1, i.e. individual pixels represent either black or white. Only a single binary bit, 0 or 1, is required to capture this for each pixel. Most formats for the realistic representation of real life images use 24 bits, 8 to represent the intensity of each of the 3 colors red, green and blue. This enables 256 different intensity levels to be represented for each of these colors, a total of 16,777,216 different tones.

File size compression

The simplest format for representing a digital image is a matrix of pixels, each pixel represented by an appropriate number of bytes to capture the required color depth and the commonly used TIFF (tag image file format) is an example of this. A TIFF file representing an image of 1000 x 1000 pixels at 24-bit color depth, requires 3 million bytes, i.e. 3 MB.

Compressed image formats such as JPEG or JPEG 2000 reduce the required image file size using mathematical techniques to store the same data more efficiently, and through the identification of data which can be discarded with minimal impact on how the image is understood by a human observer. The term 'lossless compression' refers to techniques which allow for the extraction or 'decompression' of the exact original image with no loss of detail.

Conversely, 'lossy compression' techniques permanently discard information, aiming to do so only for information which is non-contributory to the overall appreciation of the image. Lossless compression techniques are typically only able to reduce image file sizes around 2-3 fold, whereas 'lossy' techniques can achieve 50-fold or higher compression ratios, albeit with noticeable artifact.

Histology as digital images

Whole slide images are distinguished by their sheer size. A typical 15 x 15 mm tissue section imaged at 0.25 µm/pixel at 24-bit pixel depth results in an uncompressed file size of just over 10 GB. The required file size may be compressed up to 30-fold using compression techniques without impact on diagnostic utility (Krupinski et al., 2012), resulting in file sizes of the order of tens to hundreds of megabytes. It is worth noting that discernable artifacts may be introduced into the images at lower levels of compression without necessarily impacting on the diagnostic utility of the image (Foran, 1997). Moreover, greater degrees of compression may be possible for non-H&E tinctorial stains than for H&E without undermining the practical utility of the image (Sharma et al., 2012).

The way in which pathologists interact with images also influences the way image data are stored. Many vendors of WSI systems use proprietary file formats, typically based around standard image compression techniques e.g. JPEG, JPEG 2000, but with additional features. Files may include the same image at multiple resolutions to support rapid zooming in and/or out as images are streamed over

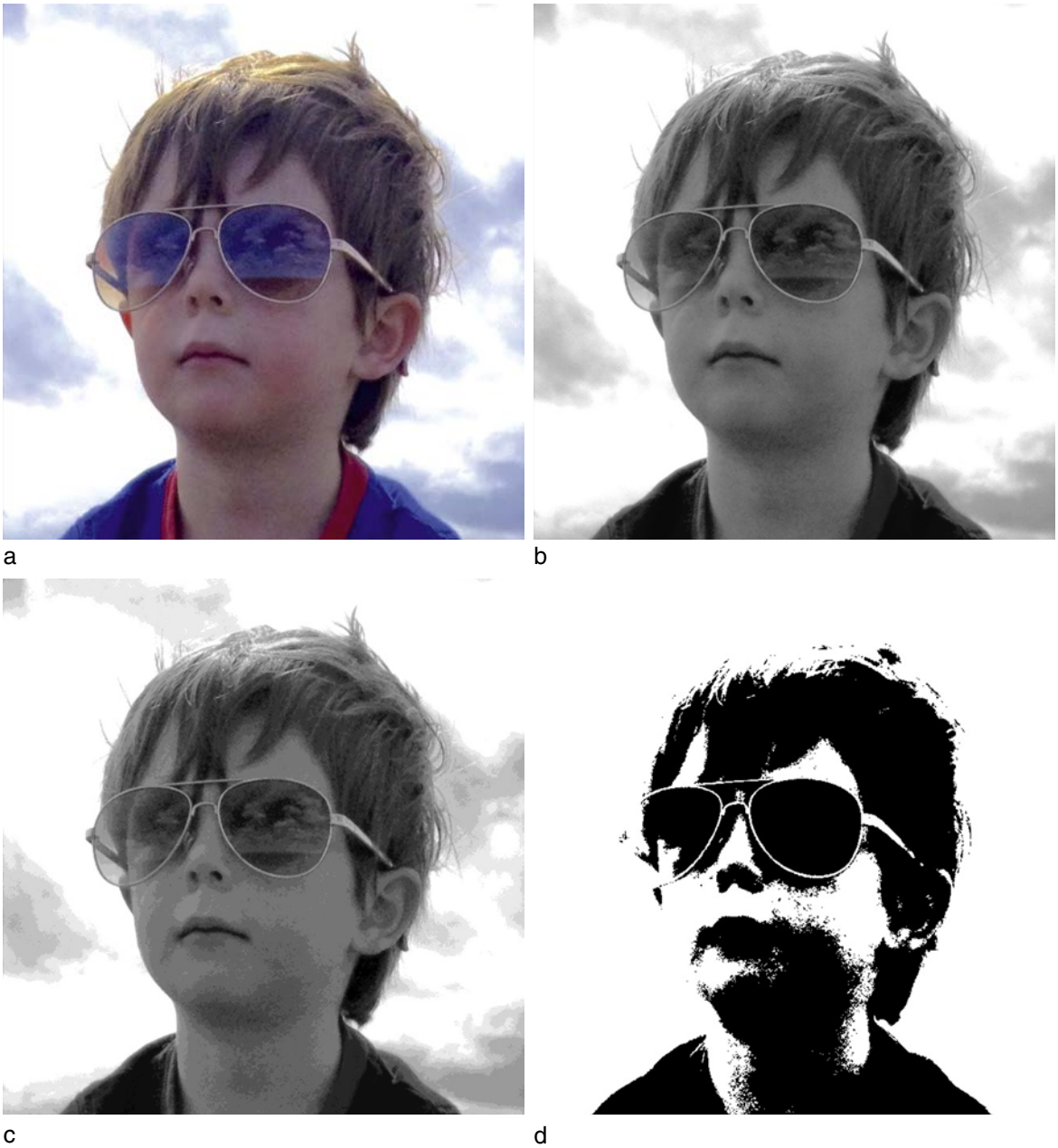


Fig. 22.1 When considering how images are represented 'perfect' fidelity may not be needed to convey meaningful information. The same original image is used throughout **a**-**d**. (**a**) A 'normal' full color image as produced by most conventional digital cameras. (**b**) The image modified to 256 shades of grey. (**c**) The image represented as only 16 possible shades of grey with a much reduced file size, but some discernible loss of image quality. (**d**) With only 2 possible values per pixel, the image is still recognizable and able to convey meaning, although it is significantly simplified and with a small fraction of the file size. (© Jonathan Bury)

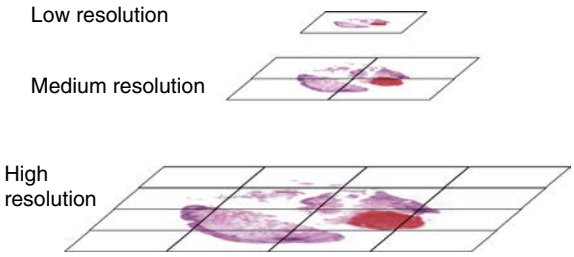


Fig. 22.2 Digital pathology file formats often use a pyramidal system. Multiple separate images are stored at different magnifications and resolutions. A pathologist viewing a low power overview image only needs to access the 'top' tier. As the magnification is increased, the relevant individual tiles from deeper tiers are displayed. This mechanism avoids the need to send entire image files across networks.

networks, i.e. pyramidal storage (Fig. 22.2). Files may also store metadata such as a timestamp, image file type, image size, pixel depth, make and model of the scanner and objective, and resolution data such as microns/pixel. Accurate metadata is particularly important if the WSI is going to be quantitatively analyzed, either manually or by an image analysis algorithm as all measurements are calculated from resolution data.

Image acquisition

A slide scanner essentially comprises an optical microscope with a mechanized stage control and focusing, coupled to a digital image capture device, usually a charge-coupled device (CCD) similar to that found in a digital camera. Additional hardware may typically include mechanical apparatus to sequentially load slides, operator controls, a visual display area and computer control hardware. The smallest single slide systems may have a footprint of only 500 x 500 mm, whilst high capacity scanners holding 200-300 slides may be 750 x 1200 mm or more. A typical 15 x 15 mm tissue area may take 30 to 60 seconds to scan using a 40x objective at 25 µm/pixel.

Systems typically use a standard 20x or 40x objective with a light path to a CCD camera. Prior to image acquisition, the slide scanner may register the sample number by reading a barcode printed on the glass slide. A scanner may also perform a low resolution overview scan to determine where the tissue is on a slide and only scan that area, minimizing overall scan time and therefore file size.

The slide is moved by the motorized stage and images are captured by the camera. Two commonly used methods of image acquisition are line scanning and tile scanning. In line scanning, the slide is moved in a linear fashion so that the camera captures strips of the image. In tile scanning, small squares of the image are captured by the camera. A strobe light source and high frame rate camera are typically employed to reduce movement, the blur artifact. With both methods, an 'image stitching' algorithm is then applied to assemble the strips or squares of image into a whole slide image (WSI). Reconstruction of a tile scanned image is computationally more complex but modern multi-core processors negate the effect this has on overall scan time.

The topography of tissue on a glass slide can vary by up to 20% of the tissue thickness over a distance of 1 mm. An image scanned using one focal plane would appear blurry in places, making it diagnostically useless. To counter this, slide scanners use an image based autofocus technique. This requires the generation of a focus map either by the operator or automatically from the overview scan image. The scanner could apply autofocus on a tile-by-tile basis in the case of tile scanning or at several points along a strip of image in line scanning. This near continuous autofocus would result in prohibitively slow image acquisition. Instead, in a trade-off between speed and image fidelity, the scanner generates a representative autofocus map, or focuses on every third or fifth tile of an image.

Special cases

Large blocks

These mega or jumbo blocks can also be scanned, but the scanner needs to be designed to accommodate larger slides. There is an increase in slide acquisition time and file size owing to the larger tissue area which needs to be scanned. Large slides may interrupt the workflow of a digital laboratory as they may require loading in separate batches to the standard size slides. One alternative to scanning

mega blocks is to create composite blocks of a sample then scan these as standard sized slides. Image stitching software can be used to create a virtual mega block from the composite blocks.

Cytology preparations

The 3-dimensional nature of a typical cytology slide presents challenges. Using a conventional glass slide, a pathologist manually adjusts the microscope to bring different 'depths' of the preparation into focus. This is not possible on a standard digital image. This problem is addressed by acquiring multiple images of the cytology slide at different focal points which are treated as a stack of 2-dimensional images, a process called z-stacking, where the z refers to the z-axis of a 3-dimensional image (x, y, z). Images must be captured in several planes of focus, with consequent multiplication of the total file size and the time taken to capture the image. When viewing these composite z-stack images on a monitor, additional image processing is required to allow the smooth transition between virtual planes of focus.

Fluorescent slides

Acquiring digital images of slides stained with fluorescent stains will typically require additional hardware, particularly a suitable light source and filters. Some vendors supply the fluorescence modules, or a dedicated scanner may be required.

Measures to ensure good quality digital images

The quality of the virtual image depends upon the production of a high quality physical slide and quality control processes relating to the scanner itself. When using a conventional light microscope, a pathologist can work around artifacts including tissue folds, wax on the coverslip, air bubbles and tissue not covered by the coverslip. A slide scanner will faithfully reproduce all of these artifacts, potentially diminishing the quality of the scanned slide.

Standard laboratory quality control procedures should ensure the production of the highest quality slides and the presence of artifacts need to be audited. Additionally, the scanner operator should re-check and if necessary clean or re-coverslip slides prior to scanning. Attention should also be paid to minimizing vibration during the scanning process. If a laboratory is situated close to a major road or rail line or other source of vibration it may be prudent to consider installing a scanner on a vibration-proof table (Fig. 22.3).

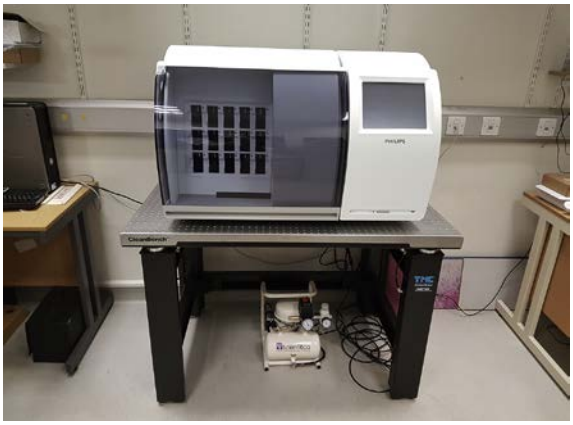
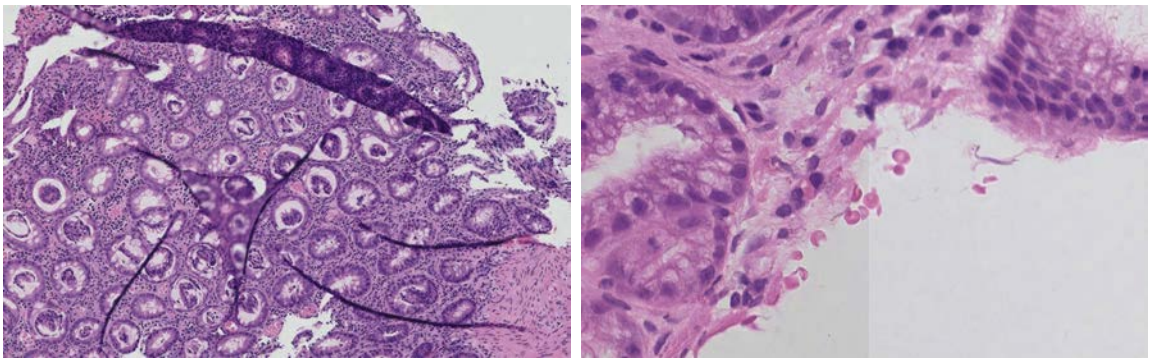


Fig. 22.3 This high-capacity high speed scanner (Philips UFS) has been installed on a vibration-damping table to minimize artifact from a railway line running very close to the laboratory. (Courtesy of Tristan Brain.)

The slide scanner should be regularly serviced and cleaned to ensure consistent lighting and focusing. A daily test slide should be scanned to assess the basic function of the scanner and detect major errors such as poor sample detection and abnormal color profiles. This procedure will also generate diagnostic information such as scanner temperature, time-to-focus and time-to-scan, all of which can create variance in digital image consistency and laboratory throughput. Color calibration of the scanner has been shown to increase diagnostic confidence and produce digital slides which are subjectively similar to slides viewed under a light microscope. This is achieved by scanning a standardized color patch affixed to a slide. The color values of this patch are known and can be compared with the on-board reference of the scanner. An adjustment to the color reproduction is then made by the scanner. This procedure is important to ensure day-to-day consistency of



a b

Fig. 22.4 (**a**) Focusing artifact where the scanner has been unable to acquire a well-focused image of the entire tissue due to folds. Using a glass slide a pathologist may be able to compensate for this by focusing up and down through the depth of the tissue but such compensation is not possible on a digitally acquired image. (**b**) Striping artifact caused by inconsistent illumination across the slide. The scanner may require recalibrating.

color reproduction by the scanner and consistency between scanners in the same department (Fig. 22.4).

Accessing and viewing whole slide images

Image streaming

The sheer size of virtual slide images raises significant technical challenges around distributing image data over networks. When slides are being viewed, software techniques are used so that only the part of the image being viewed at any one time is passed over the network, requiring only a fraction of the data transfer.

This process is referred to as streaming. The same approach is used in viewing films over the internet – rather than sending an entire movie file of perhaps 4 GB, only a fraction of the image data is sent at a time. A pyramidal image format assists with the image streaming process, with deeper subsections of the image transmitted as the pathologist increases magnification. A similar mechanism is used for the Google Earth image viewing system, in which a single low resolution image of the planet is first sent, with subsequent image data for higher magnification views of regions of interest sent in response to the user's selection of these areas. The rapid transmission of image data is achieved by only sending the area of the pyramid which is currently being viewed and potentially the surrounding image tiles. This allows smooth transition between magnifications and when panning without preloading the entire file, which would otherwise require a prohibitively large amount of network bandwidth.

Client software

This term refers to the software used by individual pathologists to request, view, navigate and manipulate WSIs, and distinguishes it from the 'server' software running on the central computer responsible for streaming the stored images to clients on demand. A key challenge in developing client software has been to provide zooming and panning in a manner which is intuitive for pathologists accustomed to physical microscopes. The speed at which the image can be refreshed is important, as a noticeable lag between operating the panning controls and the ultimate refreshing of the image will impact on the usability of the system.

Many manufacturers have developed interfaces which only require a web browser to view and navigate images, although relatively fast computers with capable graphics cards are required. Some interfaces incorporate sophisticated caseload management and

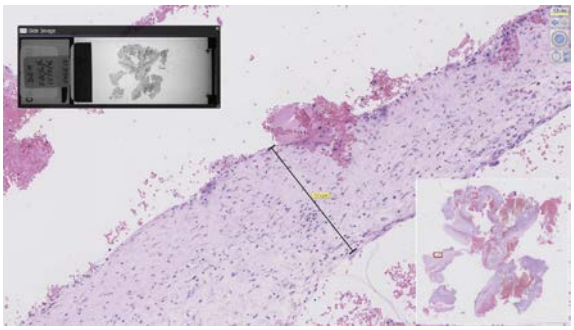


Fig. 22.5 This simple digital pathology viewing interface includes a slide overview (bottom right), as well as a view of the whole slide including the label (top left) so that identifiers on the slide label can be checked. Some image navigation controls are visible at the top right and tools for measuring, for example, can be invoked by keyboard shortcuts or mouse controls (Hamamatsu).

report writing tools which can be integrated with the laboratory information management system (LIMS). Others provide the core image viewing functionality only, which may be all that is required for many applications (Fig. 22.5).

Hardware installation and image file storage

Installation of a digital pathology system may be relatively straightforward for a small standalone scanner for which low slide volumes are anticipated. Even for such a simple arrangement however, adequate space, computer network access, a power supply (ideally dedicated and uninterruptible) and a robust table to accommodate the scanner will be required. The initial image scanning process generates particularly high data transfer requirements, far higher than the subsequent viewing of the images. For this reason, the network connection between the scanner hardware and server hardware should be as fast as possible (e.g. 1 Gb/s) and there may be a benefit to locating drives physically close to the scanner. Where higher slide volumes and large scale digitization is anticipated, multiple scanners may be needed to achieve the required throughput. Multiple units of rack-mounted server space, with fast networking connections, routers and adequate cooling will also be needed.

Operational problems with the system may be resolvable, or at least diagnosable, through remote network access from the supplier's technical support team. Arrangements for such access should be established in advance, taking into account institutional network security policies.

Image storage arrangements

Hard disc drives offer high capacity and rapid access. Tape drives have fallen somewhat out of fashion in recent years, but the current generation of tape storage systems offer high storage capacities at low prices. The LTO-7 (linear tape open, 7th generation) format provides 6 TB of uncompressed storage on a single cassette measuring 102 × 105.4 × 21.5 mm. The trade-off for tape systems is that files are inevitably slow to retrieve. One strategy for minimizing the costs of a large clinical slide archive whilst maximizing its performance is to specify different tiers of storage. The initial acquisition of a slide image requires the highest drive speeds as the entire image file is captured. Unreported images should be stored on high performance disks with the fastest available data-read times on servers able to deliver images to multiple pathologists at the same time. However, once reported, the images for these cases could be moved to a cheaper, less highly specified medium. The subset of slide images which need to be revisited, e.g. for multidisciplinary team/clinical review can be accessed, but with some minor lag in image availability.

If governance or regulatory frameworks mandate longer term archival storage of images, then tape drives may provide the most cost-effective solution. This is analogous to the arrangements for glass slide filing in most laboratories, where the most recent cases are in immediately accessible storage occupying valuable laboratory space, whilst older cases may be stored in cheaper offsite space, trading retrieval times for cost. Consideration should be given to the location of the data storage system. If it is accepted that a digital image must be held for a period of time for quality assurance purposes, then thought must also be given to how that storage is backed up. Primary storage is likely to exploit redundant array of interchangeable discs technology (RAID).

In the RAID model a cluster of drives store multiple (2 or 3) copies of each image file across multiple individual disc drives. Should an individual disc fail, that unit can be replaced and the affected files restored from one of the other copies. This does not protect against catastrophic data loss due to, e.g. fire or flood. A robust approach to long-term storage is likely to involve multiple tape copies in different physical locations. The final cost will be considerably higher than the simple cost of drives and tapes. Any storage medium has a finite lifespan and real-world data storage solutions need to allow for regular migration of data onto new drives or tapes etc. to ensure data integrity.

Applications

Non-diagnostic applications

Education & training

Some of the earliest applications for WSIs were as educational resources. In an educational context, the speed of image acquisition is less critical than it may be in a clinical setting, so the longer scan times of early scanners (measured in minutes) are acceptable. Image quality may also be less critical. The ability to annotate images is particularly useful as significant features can be highlighted more clearly than using the traditional 'ink dot on the slide'. Many institutions have established large digital slide archives for educational purposes, and software can be used to enrich such resources with assessment questions, links to text etc.

Research, including image analysis

Digital pathology technologies can aid a research pipeline in two ways. Firstly, the file storage and viewing applications described above can be used to organize macroscopic digital photographs, whole slide images and tissue microarrays. This is useful in multicenter studies with central pathology review. Additionally, tissue banks can add a virtual slide bank to their resource, allowing easier collaboration. This approach has been pioneered by the Cancer Digital Slide Atlas, an element of the Cancer Genome Atlas (http://cancergenome.nih.gov/).

The second use of digital pathology in research is the development and application of image analysis techniques. Algorithms can group pixels within an image by similarity to their neighbors, identifying nuclei, stroma, architectural features or even specific cell types, e.g. lymphocytes. Further manipulation of these groups allows objective calculation of staining which can be useful in quantitatively assessing hyperchromasia in standard stained slides or antigen expression by immunohistochemistry. This creates the opportunity to measure binary or categorical features, e.g. low or high grade dysplasia, on a continuous scale with a potentially higher degree of precision and reproducibility.

Image analysis algorithms can be combined to measure multiple features in an image. Many of these features cannot easily be assessed by eye, e.g. the orientation (in degrees) of nuclei relative to one another, the distance between nuclei, the precise percentage of tumor and stroma, the degree of nuclear membrane irregularity and the absolute nuclear area. Each of these features can be measured on a continuous scale and given varying weight in statistical models which predict clinical variables, including response to treatment, survival and tumor recurrence (Beck et al., 2011). This technique has been called 'tissue-omics', suggesting a kinship with other big data techniques such as genomics, proteomics and metabolomics. It is the combination and integration of data from these sources which provide the greatest potential for important research findings. However, an enormous amount of data is generated in each of these approaches and storage, transmission and analysis become the limiting factors.

Quality assurance

Diagnostic external quality assurance (EQA) schemes involve the circulation of sets of slides to pathologists to ensure diagnostic uniformity. Where large numbers of pathologists are involved, multiple sections from the same tissue blocks may be required precluding the use of small biopsies or cases with only focal pathology. The use of digital slides avoids this problem allowing integration with web-based systems for collating diagnoses. Digital slides are now widely used and accepted in diagnostic EQA schemes in the UK.

The technology is currently enabling, e.g. approximately 500 pathologists to regularly participate in the UK Bowel Cancer Screening Programme scheme, with participants in the UK, Eire, Slovenia and the Netherlands.

Diagnostic applications

The improvement in scan times and image quality is allowing digital pathology to be used as a viable option for the primary diagnosis in routine clinical practice. As noted at the start of this chapter, digital pathology advantages include the ability of the technology to seamlessly, near-instantaneously move images from one location to another, and the positive impact on slide filing, archiving and retrieval. The balance of cost against benefits therefore favors applications where it is important to move slide images rapidly and/or where a digital approach tangibly reduces the need for filing and retrieval.

Remote intraoperative diagnosis

Intraoperative cryostat (frozen section) diagnosis has been one of the first applications where digital pathology has reached clinical practice. This has been driven by geographical arrangements, specifically the scenario where laboratories are not located at the same site as the operating theatre. Early approaches to this problem employed remote microscopes, i.e. a video camera linked to a microscope, with stage controls operated by a distant pathologist, or transmission of still images of areas of interest taken by an on-site technician and, e.g. emailed to the pathologist (Fig. 22.6).

Whole slide imaging avoids the complexity and time-lag of a remote microscope and enables the entire slide to be seen rather than a pre-selected region of interest. Studies have demonstrated comparable accuracy to that achieved using light microscopy (Bauer et al., 2015; Evans et al., 2010). One practical point to note is that ensuring uniform section thickness and avoiding tissue folds is particularly difficult with cryostat preparations, and this can lead to problems acquiring a well-focused image. Z-stacked images capturing multiple focal planes help overcome this but may significantly increase scan times which can be unacceptable in the intraoperative context. Care must also be taken to avoid excess mountant coming into contact with the scanner objective lens (Fig. 22.7).

Fig. 22.6 This Hamamatsu scanner is used for capturing images for intraoperative frozen section diagnosis. The system holds just 6 slides but offers fast scan times which is appropriate for this application.

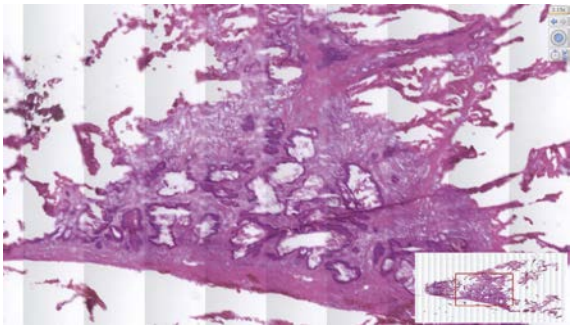


Fig. 22.7 This image is part of a whole slide image acquired intraoperatively. During the scanning process the objective came into contact with excess mountant on the slide and the resultant image is unsuitable for diagnosis.

It is also necessary to have scientific staff at the site of surgery. These personnel can examine and then dissect the specimens. This means for example, breast duct or bronchial resection margins are dissected and oriented appropriately, or tumor masses within lung wedges are sampled confidently.

Second opinion

The ability to swiftly and easily seek a second opinion is an often-cited application where digital pathology may be of benefit. As above, the speed at which the digital image is viewable by a pathologist

is an advantage. There are also savings on packing and postage. Digital image transmission also avoids the situation where a slide is needed for clinicopathological review and discussion, but is instead in-transit to/from the second opinion site. Beyond these logistical benefits, digital slides can be annotated on screen to draw attention to particular areas of interest or diagnostic relevance. It is also possible for geographically separate pathologists to confer by telephone whilst looking at the same image on screen, rather than needing to be together at a multi-headed microscope.

Multidisciplinary team (MDT) meetings and clinicopathological conference (CPC)

Locating, retrieving and refiling slides for regular MDT/CPC meetings, places a considerable burden on many laboratories. In a conventional 'glass slide' laboratory, it may be possible to selectively scan slides from cases likely to be discussed if they are identified as such by the reporting pathologist, or flagged on receipt in the laboratory. This selective scanning however may simply introduce additional operational complexity and delay. Using digital pathology to support MDT/CPCs may be more efficient within the context of a fully digital laboratory process. Once flagged as being needed, digital images allow easy annotation of histological features of interest which can speed up case review and enable salient features to be easily displayed to the clinicians. This digital data handling is already well established in radiology.

Clinical quantification

Measurements of dysplasia, immunohistochemical staining, percentage of tumor/stroma and glandular complexity are examples of features which a subjective observer, the pathologist, must objectively ascribe to recognized categories of histopathological features. This leads to measurable intra- and inter-observer variation. At a clinical level, image analysis can perform tasks which pathologists do already but with the promise of greater precision, reproducibility and efficiency.

Many image analysis systems exist which are aimed at improving the accuracy and reproducibility of HER2 scoring in breast cancer. At least one of these is FDA approved for clinical use. Early work suggested that an image analysis approach may resolve equivocal HER2 scores reducing the need for secondary FISH analysis. The problems with validation and reproducibility between laboratories however, have hampered widespread use largely due to the variation in staining intensity between, and even within the laboratory. This requires frequent recalibration and validation of algorithms which ameliorates time and efficiency savings, introducing the possibility of error in a supposed 'objective' system. Additionally, the handing over of such an important task to a 'black box' may be viewed with unease by clinicians and patients alike.

Algorithms have also been developed which either assess the stroma: tumor ratio or count tumor nuclei. These are important tasks when submitting formalin fixed paraffin wax embedded (FFPE) blocks for mutation analysis, e.g. in lung cancer. Currently these algorithms have only been used for quality control and benchmarking when performed by a pathologist. In this situation, the image analysis software produces an objective measure against which pathologists can be assessed. By increasing the use of digital slides and a digital workflow, it can be seen how automated results from digital image analysis could be integrated into a report. In this situation image analysis may become a tool which can be deployed on demand and augment the performance of pathologists and the laboratory.

Whole laboratory digitization

Many pathologists have gained familiarity with digital images through involvement in research, education and quality assurance schemes, but despite the advantages and maturity of the technology there are few examples of entire laboratories which have adopted a fully digital approach. There has been a tendency to attribute this lack of adoption to pathologists being unwilling to work from digital images. This is to some extent reasonable, as some early systems offered suboptimal image quality and unresponsive user interfaces. The lack of penetration into routine practice however, may have less to do with the images themselves, but instead that there is little benefit to simply digitizing images for the sake of it.

A fundamental change occurred with the emergence of pathologist workflow management systems. Contemporary digital pathology clinical interfaces borrow heavily from lessons learnt in radiology, and offer far more functionality than simply the ability to view a digital slide image. The user interface can be tightly integrated with the LIMS, meaning previous reports and images for a patient under consideration can be accessed. Cases can be assigned to individual pathologists with varying levels of prioritization and cases can be flagged for MDT, educational discussion etc. The availability of these features makes digital pathology a far more compelling proposal than viewing images on-screen for no reason other than it being technically possible. Digital workflow has demonstrated measurable improvements in productivity (Haroske and Moerz, 2016). The barrier to adoption is now more to do with the high capital costs of scanners and data storage than the acceptability to pathologists. At the time of writing a typical high-speed, high capacity scanner may cost between $100,000-150,000. Nevertheless, some laboratories are already scanning high proportions of their workload (Thorstenson et al., 2014; Baidoshvili, 2015). The practical issues surrounding the transition to a fully digital workflow are discussed in the 'Future development' section at the end of this chapter.

The digital pathology workstation

The digital pathology workstation comprises at least two computer monitors with separate interfaces on each. The first monitor displays a virtual microscope which allows familiar controls such as panning and zooming. This monitor should be of medical grade and calibrated to consistently reproduce histologic color profiles. Calibration with a standardized test object has been shown to improve diagnostic confidence and produce color profiles subjectively closer to equivalent glass slides. The virtual microscope allows improvements in workflow efficiency. Dedicated software, e.g. that used with the Leeds Virtual Microscope (Randall et al., 2014), will move pixels quickly enough so the whole slide image can be panned and zoomed without artifacts or pixelation occurring. Small 'thumbnail' images of each slide in a case allows ease of movement between slides and rapid comparison of non-sequential slides. Virtual microscope software also allows slide annotation to identify regions of interest (ROI), which are useful for teaching sessions or consultation with a colleague. These regions can also be extracted and appended to a report.

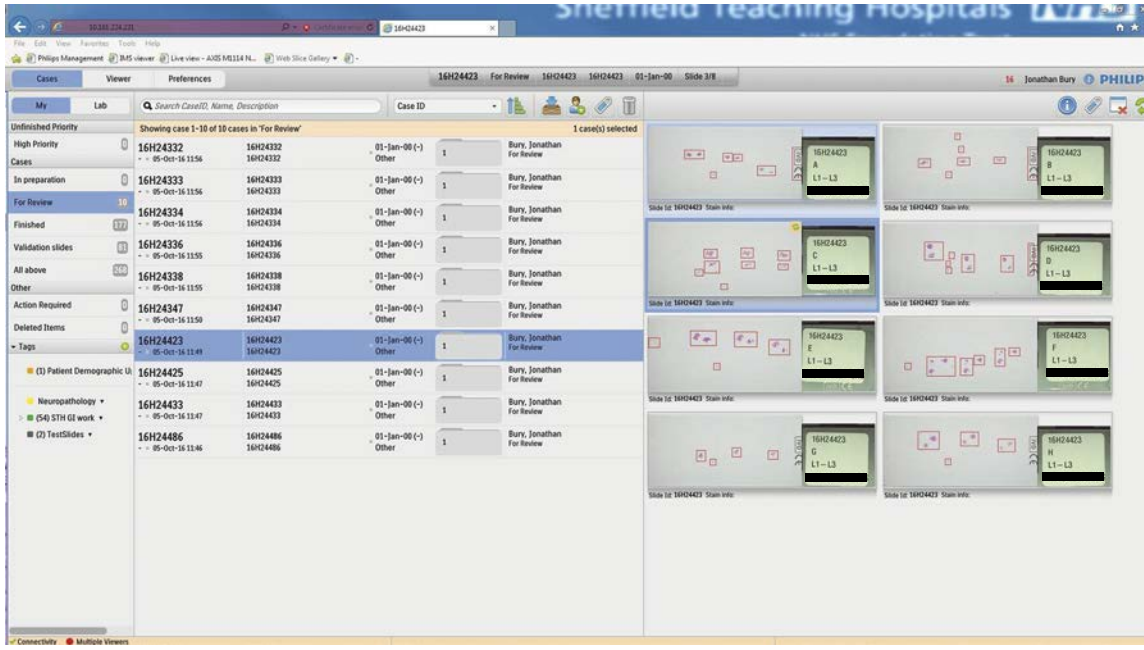
The second monitor of the workstation provides an interface for the LIMS or image management software (IMS). At its simplest level, this displays a list of cases and their status, e.g. to be viewed, report in progress or report authorized. When scaled up over an entire department this interface facilitates distribution of work and real-time monitoring of case turnaround time. Additional functionality can include instant sharing of cases and the tagging of cases with labels which automatically add the case to an MDT meeting agenda or a teaching set.

The virtual microscope and the LIMS/IMS interfaces have the potential to improve efficiency and safety within a pathology department but their design must facilitate ease of use (Fig. 22.8).

Validation and regulatory issues

The validation of a WSI system is essential prior to its use for routine diagnostic work. A validation study aims to demonstrate that a diagnosis from a digital slide has at least the same accuracy as that from a glass slide. The Digital Pathology Association and the College of American Pathologists have both published guidelines on the conduct of validation studies. These guidelines state that the WSI system should be validated in its entirety in a real-world clinical setting and separately validated for each new clinical application. Each slide is viewed by the same pathologist using a light microscope and a digital microscope. A washout period, the time between digital and glass slide review, of up to four weeks is recommended to reduce recall bias. It has been suggested (Campbell et al., 2015) that a longer washout period may be necessary as effective recall of cases is common, even after a period of months, between glass and digital slide review.

A sample size of between 60-100 cases is recommended for each validation study. Most high quality recently published studies have greatly exceeded this number. The largest study to date used a sample



a

b

Fig. 22.8 A clinical workflow oriented interface. As well as enabling individual images to be viewed, connection to LIMS enables a variety of pathologist 'workflow' functions to be accessed. A split screen is provided. The workflow (**a**) shows those cases which are assigned to a particular pathologist and different priorities can be assigned. Cases can be assigned to different folders, e.g. cases of educational value or cases for team discussion. Cases are marked as 'finished' once reported. The image (**b**) displays the slide currently being viewed. Measurement and annotation tools are available and a slide overview is provided (top right). Documents associated with the case, e.g. the request form or macroscopic photographs may be accessed through the same interface. Reports may also be typed directly into this user interface and authorized (Philips).

size of over 3,000 cases, totaling 10,000 slides. Ideally, each slide should be reviewed in digital and glass format by two pathologists, allowing calculation of inter/intra-observer agreement respectively. In practice this often creates an unfeasible volume of work in addition to routine reporting, and a hybrid of inter- and intra-observer agreement is used to give overall concordance between the glass and digital diagnosis. Any non-concordant cases should have a ground truth diagnosis made by consensus.

Examples of validation studies

Following the development of whole slide scanners in the late 1990s, the first validation studies of digital pathology took place in the early 2000s (Gilbertson et al., 2006). A recent systematic review found 38 validation studies (5312 total cases) from 2006-2015 (Goacher et al., 2017). The weighted mean percentage diagnostic concordance was 92.4% with a kappa diagnostic agreement of 0.75 indicating substantial agreement between digital and glass diagnoses. A trend towards higher quality study design over time was observed which may reflect the introduction of the College of American Pathologists (CAP) and Digital Pathology Association (DPA) validation study guidelines in 2013.

Subsequent to this systematic review, Snead et al. (2016) showed digital pathology (DP) diagnosis to be of equivalent accuracy to light microscope (LM) diagnosis in a large single center validation study. A clinically significant discrepancy between DP and LM occurred in 0.7% of cases. It is worth noting that in nearly half of these cases the ground truth diagnosis, arrived at by consensus, lay with the DP diagnosis. The authors also commented on potential benefits to workflow when using digital pathology, e.g. easier measurement of margins and tumor size, and easy access to and sharing of cases. Several smaller validation studies demonstrated similar equivalence between LM and DP in individual specialty areas such as gynecological and colorectal pathology.

Regulatory frameworks and standards

Whole slide scanners are classified as medical devices and are subject to regulatory frameworks. Several vendors have been granted the Conformité Européenne (CE) mark for digital slide scanners which enables these to be used within the European Union for all relevant applications, including primary diagnosis. A similar situation exists in Canada where a Class II Medical Device License has been granted to two digital pathology vendors. This classifies scanners as moderate risk devices and allows these systems to be used for primary diagnosis.

In the USA, digital slide scanners are currently classified as Class III medical devices by the Food and Drug Administration (FDA). A class III device is considered to be of substantial importance in preventing impairment of human health or present a high risk of causing injury or illness. This means that pre-market approval (PMA) is required before a scanner can be used for primary diagnosis. Following negotiation between the Digital Pathology Association and the FDA, vendors may now make a de novo application for a scanner to be classified as a class II device. This pathway is shorter and less costly than the PMA route and may pave the way for the use of digital pathology in primary diagnosis in the USA.

File storage

The UK's Royal College of Pathologists (RCPath) currently recommend that glass slides are held for 10 years, whilst paraffin wax blocks must be held for 30 years. This is to ensure that the pathology remains available should subsequent review be required. The RCPath's 2014 guidelines recommend that digital images used for primary diagnosis are retained for audit purposes for 10 years, or at least two cycles of laboratory accreditation, effectively 8 years under the 4 yearly clinical pathology accreditation (CPA) regime or potentially just 2 years under the annual ISO system.

A laboratory producing 1000 slides per day may choose to store digital images for 1 month for initial diagnosis and MDT/CPC review, prior to deletion. Storing each digital image file for 10 years from the date of creation however, would increase the storage requirement by a factor of 120, pushing the storage requirements of a large laboratory towards petabyte (10^{15} bytes) volume, with significant cost implications.

Future development

Image quality and scan speeds have reached the point where routine digitization of all slides, even in a large laboratory can be contemplated. At the time of writing, digital slide scanners are still relatively novel pieces of equipment and they are certainly not routine capital purchases for the vast majority of laboratories. Nevertheless, akin to desktop computers, it is likely that they will/must become commonplace pieces of laboratory hardware.

The typical scanner size, in terms of the number of slides which can be loaded at once, may evolve as experience directs the balance between the convenience of a single large machine versus the resilience and flexibility of using a pool of smaller machines. The requirement for fluorescence and jumbo slides will also need to be catered for. There is a possibility to integrate staining, cover-slipping and scanning processes into a single piece of hardware. These changes are perhaps best seen as refinements and evolution, rather than fundamental changes.

More significance will be attached to the software and technical infrastructure which stores and distributes images and supports the pathologist's workflow practice. Vendors are less likely to highlight the optical quality or scan speed which will be taken for granted. Greater emphasis will be placed on demonstrating how software tools can improve the workflow both of individual pathologists and the laboratory as a whole. There may be convergence with other medical image handling systems as ultimately, whole-slide images are just one more form of image data handled in healthcare, alongside radiology images, scanned documents and endoscopic photography.

A fully digital pathology department fundamentally changes how a pathologist interacts with their workload. For example, trays of slides no longer need to be collected or delivered to an individual pathologist's office, slides from historical cases do not need to be retrieved from archival storage and cases required for MDT meetings or teaching sessions cannot be lost or left in an obscure location. Additionally, digital slides can be viewed simultaneously by multiple doctors allowing synchronous review by trainees and consultants, or by two consultants in cases which require double reporting or second opinions. These changes in workflow require an interface which facilitates the viewing and reporting of digital slides.

Such whole laboratory digitization presents considerable technical challenges. Substantial investment in servers, network infrastructure and suitable viewing stations are required. The amount of data generated each day may be in the order of tens or hundreds of gigabytes, an order of magnitude higher than even the largest of radiology departments. A single high capacity scanner will take time to scan a full load of approximately 300 slides, even at 30 seconds to 1 minute per slide, introducing a delay. An alternative configuration may be to use two or more medium capacity scanners, perhaps each holding 120-150 slides and operating in parallel. This arrangement also has the advantage of affording some resilience in the event of scanner breakdown. The manpower required to load and unload slides to and from a scanner may be offset by the time saved distributing slides around a laboratory and their subsequent collection.

Realizing the benefits of a digital workflow

From the description above it should be clear that the technology required to enable a digital slide workflow across a laboratory's entire operation is available. However, simply investing in the equipment is unlikely to be sufficient to realize the potential benefits. The successful deployment of new technology into any environment requires not only that the technology be a reasonable 'fit', but also that some modifications will be needed to that environment so that the potential of the new technology is best exploited. Some specific issues of relevance to a whole-laboratory digitization program may be as follows.

Integration with existing laboratory management systems

As the pathologist's interaction with histological images moves from the microscope to the computer,

one useful concept is the distinction between a LIMS driven workflow and an IMS driven workflow. In a LIMS driven approach, a pathologist's primary point of interaction is with the standard hospital information system which may present the pathologist with a worklist of cases for reporting, tools for viewing previous patient reports and a mechanism for creating a report on the case currently under consideration. This interface could be expanded to trigger the presentation of a whole-slide image as required.

The LIMS is the pathologist's primary point of interaction, much as it might be in a non-digital environment, and it is this which 'drives' the presentation of digital slide images. Conversely, it may be that the image management software instead serves as the pathologist's primary point of interaction. Worklists, or other case management tools, can be created in the IMS, which may communicate 'behind the scenes' with the laboratory information system enabling reports to be created and authorized (signed-out), or other patient information such as previous reports to be accessed.

A pathologist's workflow involves selection of a case of interest, review of the clinical details, macroscopic description, viewing the slide(s) and then the drafting and authorization of a report. Additional steps may include the review of previous reports for that patient, or review of a previous slide, as well as ordering additional stains. Cases may be shared with colleagues or set aside for educational purposes and MDT meetings etc. One model of workflow integration would use the LIMS as the main focus of the pathologist's interaction, with the digital image server prompted by the LIMS to display images of interest. An alternative model places the digital image management system (IMS) at the forefront of the pathologist's interactions, with messages passed to and from the LIMS in the background as required. These models are referred to as LIMS driven and IMS driven workflows respectively. The choice of approach is to some extent arbitrary. Laboratories with a limited LIMS interface may prefer an IMS driven model, which may also be appropriate where pathologists in one department are required to review material from various source laboratories with different LIMS. Laboratories with a well-developed and operationally efficient LIMS may opt to retain that interface at the center of their workflow.

Barcoding of slides

This may require investing in new labelling equipment and other adjustments to the laboratory processes. The barcode must contain sufficient information to uniquely identify each slide. A variety of barcode formats exist. One-dimensional barcodes comprise a series of parallel black and white lines, whilst 2-dimensional formats, e.g. the quick response (QR) code is an array of black and white squares (Fig. 22.9).

Report creation

If pathologists are interacting directly with slide images on screen, it may be logical for them to enter and sign-out the final report concurrently where possible, perhaps using voice recognition software with a standardized 'canned text' phrase or a reporting template. In many laboratory workflows however, entry of the specimen details, clinical details and the macroscopic description elements of the final report does not take place until the dictated microscopy and final interpretation is typed. Entry of clinical details, macroscopic description

Fig. 22.9 This slide bears a 2-dimensional barcode. This encodes data which uniquely identifies the slide and includes patient identifiers and details of the stain used. Use of such barcodes on slides is essential in high volume slide scanning applications to avoid time-consuming and error-prone manual image annotation.

etc. would have to be rescheduled so that it preceded microscopic reporting if pathologists were to be able to sign-out as soon as the slide images are inspected.

Adoption of paperless requesting and reporting

The benefits of digital distribution of slides are lost if paper request forms must be distributed to pathologists to enable them to report, e.g. to see the clinical details, etc. It may be that the initial clinician's request can be made electronically, or that paper request forms are scanned on receipt with the scanned request form image presented to the pathologist as slides are viewed. Establishing institution-wide paperless requesting or routine scanning of paper request forms by the laboratory are both significant undertakings.

Staff training and reprofiling

Whole-laboratory digitization offers savings in terms of staff time spent on sorting, filing and retrieving slides as well as potential saving on slide storage. However, this will be offset by the requirement to load and unload slide scanners and there will be a need for equipment troubleshooting and maintenance with significant IT support.

Pathologist workstations

Routine institutional software updates, e.g. security and software upgrades may conflict with the requirements of digital slide viewing software, raising the possibility that the digital pathology workstations need to remain separate from the pathologists' standard PC used for email and other routine office tasks.

Pathologist training

This chapter has not addressed whether reporting from on-screen images is acceptable to pathologists, although the large validation studies cited above all suggest that the technology is effective. Training in the use of the software chosen will be required, and it may be for accreditation purposes that this training should be documented and repeated at intervals.

Application of specific validation studies

As noted above, validation is required for each application of a digital slide, e.g. general gastrointestinal pathology or neuropathology. Ongoing audit of each case type where digital reporting may pose specific challenges, e.g. grading of dysplasia or identification of organisms such as *Helicobacter* may be required. Separate validation studies may also be required for special stains, immunohistochemistry, frozen sections and cytology preparations.

Summary

Although the technical principles of digital image handling are well established in other fields (e.g. radiology), adoption of the technology in histopathology will inevitably be challenging. Existing laboratory processes will need to be adapted so that the full potential of the new technology can be made. Guidance on governance arrangements for instance on slide retention will need to be updated and pathologists and biomedical scientists will see changes to the nature of their work. There is uncertainty surrounding how such extensive changes can take place, particularly given the high costs of the equipment involved. Nevertheless, digital pathology is likely to fundamentally impact on histopathology laboratory operations over the next decade.

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Diagnostic Appendices

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Appendix I

Traditional methods

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Introduction

The aim of this appendix is to include discussion and methodology from the chapters Lipids, Proteins, Nucleic Acids and Enzyme Histochemistry which were included in previous editions of this book. These technologies have largely been superseded in the modern laboratory but are still used in teaching courses worldwide. For a full discussion and omitted methods please refer to the sixth edition of this text.

Lipids

These methods have an application in diagnostic pathology for nervous system disorders. The myelin sheath is particularly rich in lipids, being composed of a compacted cell membrane which is a lamellar structure of cholesterol and phospholipids. The lipid demonstration methods can aid in the diagnosis of demyelination and the lipid storage disorders.

Lipids may be defined as any one of a group of fats or fat-like substances. These fats include:

- True fats – esters of fatty acids and glycerol.
- Lipids – phospholipids, cerebrosides and waxes.
- Sterols – cholesterol and ergosterol.
- Hydrocarbons – squalene and carotene.

Classification

Lipids may be classified as a mixed group of substances with the common characteristics of solubility in organic solvents and insolubility in water. They can be organized as simple lipids, compound lipids or derived lipids.

- Simple lipids: esters of fatty acids with alcohols, including fats, oils and waxes. Fats are neutral esters of glycerol with saturated or unsaturated fatty acids. Oils may be similar to fats but are liquid at room temperature. Waxes are esters of higher alcohols with long-chain fatty acids. Simple lipids are usually found in the body as energy stores in adipose tissue. Waxes are usually found in plant and some animal species.
- Compound lipids: usually consist of a fatty acid, an alcohol a phosphoric acid and a nitrogen base. These can be found in the brain and central nervous system.
- Derived lipids: fatty acids which can originate from the simple and compound lipids by means of hydrolysis. Cholesterol, bile acids, sex and adrenocortical hormones are examples.

Fixation and microtomy

The common method for demonstrating tissue lipids is with fresh frozen (cryostat) sections. Some degree of fixation may be necessary so that lipids and the sections themselves are able to withstand the potentially destructive or solvent effects of histochemical reagents. The only reagents which truly fix lipids are osmium tetroxide and chromic acid, but both these greatly alter the chemical reactivity of the lipids. Frozen or cryostat sections are required for lipid histochemistry because routine processing for paraffin wax and resin sections will result in the extraction of all but a few protein-bound lipids from

the tissue. Although lipids are not strictly fixed by formaldehyde, they are better retained in a section when the supporting matrix of tissue proteins has been fixed.

Fat stains and the Sudan dyes

Lipids which exist as fats, namely the oily and greasy hydrophobic lipids, have an affinity for the Sudan dyes. For many years a wide range of these compounds has provided almost the sole means of staining lipids.

Oil Red O in dextrin (modified by Churukian (2000))

Fixation

Fresh frozen or NBF.

Sections

5 µm mount on slides, air dry.

Solutions

Oil Red O solution

Oil Red O	0.5 g
Absolute isopropyl alcohol	100 ml

Allow to stand overnight.

Dextrin solution

Dextrin*	1 g
Distilled water	100 ml

*Bacteriological grade or Type III from Sigma, from corn. Dextrin is hydrolyzed corn starch. VWR Scientific also has small quantities, must be most soluble form of dextrin.

Working solution

Stock Oil Red O	60 ml
Dextrin	40 ml

Mix and allow to stand for a day or more. Stable for months, filter before use.

Method

1. Place slides directly into filtered 0.5% Oil Red O in dextrin. Stain for 20 minutes, rinse with running water briefly.
2. Counterstain with Gill II hematoxylin for 20–30 seconds. Rinse with water, blue, coverslip with aqueous mounting media.

Results

Lipids	brilliant red
Nuclei	blue

Standard Sudan black B method for fats and phospholipids

Fixation and sections

Cryostat sections post-fixed in formal calcium; short fixed frozen sections; unfixed cryostat sections (preferred).

Method

1. Rinse sections in 70% ethanol.
2. Stain for up to 2 hours in saturated Sudan black B in 70% ethanol.
3. Rinse in 70% ethanol to remove excess surface dye and wash in tap water.
4. Counterstain nuclei with Kernechtrot for 2–5 minutes.
5. Wash well and mount in glycerine jelly.

Results

The standard Sudan black procedure stains unsaturated esters and triglycerides *blue-black*. Some phospholipids appear gray and those in myelin exhibit a *bronze* dichroism in polarized light.

Bromination enhances the reaction of these lipids and in addition stains lecithin, free fatty acids and free cholesterol.

Note

The Sudan black solution should not be oversaturated or sections will be covered in a fine deposit. Fixation enhances the staining of phospholipids (present in all tissues) and this is unwanted in general use, thus unfixed sections are preferred.

Cholesterol

Perchloric acid-naphthoquinone (PAN) method for cholesterol (Adams, 1961)

Fixation and sections

Formal calcium fixed frozen section; cryostat sections post-fixed in formal calcium.

Preparation of reagent

1:2 naphthoquinone-4-sulfonic acid	40 mg
Ethanol	20 ml
60% perchloric acid	10 ml
40% formaldehyde	1 ml
Distilled water	9 ml

Mix and use within 24 hours.

Method

1. Air dry sections onto slides.
2. Treat with 1% ferric chloride for 4 hours.
3. Wash well in distilled water.
4. Carefully paint the sections sparingly with the reagent using a soft camel-hair brush. (Note: wash the brush thoroughly with water after each use, and dry.) Heat them on a surface at 70°C for 1 or 2 minutes, until the color develops. The sections are kept moist by gently replenishing the reagent from time to time.
5. Place a drop of perchloric acid on a cover glass and lower section into position.

Results

Cholesterol and related steroids	blue

Sphingomyelin

Sodium hydroxide-ferric hematoxylin/DAH method

Fixation and sections

Formalin fixed sections, preferably mounted on chrome gelatin subbed slides.

Method

1. Treat sections with 2 M sodium hydroxide for 1 hour at room temperature.
2. Wash gently but thoroughly in a large volume of water.
3. Rinse in 1% acetic acid for 5 seconds.
4. If section has become detached from the slide, remount it and proceed with the ferric hematoxylin method as described for phospholipids in previous editions of this text (Elleder & Lojda, 1973).

Results

Sphingomyelin	blue

Cerebrosides

Modified PAS reaction for cerebroside (Adams & Bayliss, 1963)

Fixation and sections

Cryostat sections post-fixed in formal calcium; fixed frozen sections.

Preparation of reagent

98% formic acid	45 ml
100 vol hydrogen peroxide	4.5 ml
Concentrated H_2SO_4	0.5 ml

Prepare an hour before use and stir occasionally with a glass rod inside a fume hood, to release bubbles of gas from the solution.

Method

1. Mount duplicate sections onto separate slides and extract one of these with chloroform methanol (2:1 v/v) for 1 hour at room temperature.
2. Deaminate both sections in 10% aqueous chloramine T for 1 hour at 37°C.
3. Wash slides vigorously and as rapidly as possible, one at a time, in a large volume of water before transferring them immediately to performic acid for 10 minutes. The washing must be swift yet thorough, to avoid swelling and detachment of sections from slides.
4. Wash well in distilled water.
5. Treat with a filtered saturated solution of 2:4 dinitrophenyl hydrazine in 1 M HCl at 4°C for 2 hours.
6. Wash well in water.
7. Treat with 0.5% periodic acid for 10 minutes.
8. Wash in distilled water.
9. Stain in Schiff's reagent for 15 minutes.
10. Rinse in distilled water and wash in tap water for 15 minutes to develop color.
11. Counterstain nuclei with Mayer's hematoxylin or Carazzi's hematoxylin if required.
12. Wash in tap water, distilled water and finally mount sections in glycerine jelly.

Results

Cerebrosides	magenta

Indicated by the difference in staining intensity between the two sections.

Sulfatides

Toluidine blue-acetone method for sulfatide (Bodian & Lake, 1963)

Fixation and sections

Post-fixed cryostat sections; formal calcium fixed frozen sections.

Reagent

0.01% toluidine blue in phosphate-citrate buffer at pH 4.7.

Buffer solution

0.2 M Na_2HPO_4	96 ml
0.1 M citric acid	104 ml

Method

1. Mount sections onto slides.
2. Stain for 16–18 hours in buffered toluidine blue.
3. Wash in water.
4. Dehydrate with acetone for 5 minutes.
5. Mount in DPX.

Result

Sulfatide deposits appear metachromatic red, brown or yellow

Gangliosides

Borohydride-periodate-Schiff (BHPS) method

Fixation and sections

Cryostat sections post-fixed in formal calcium; frozen sections of fixed tissue.

Method

1. Destroy existing aldehyde groups (endogenous or from formalin or glutaraldehyde fixation) by reduction with 0.1 M (0.38%) sodium borohydride in 1% disodium hydrogen phosphate for 1 hour at room temperature.
2. Wash thoroughly in distilled water.
3. Oxidize with 1.2 mM (0.03%) sodium periodate for 30 minutes at room temperature.
4. Wash twice for 5 minutes each time in distilled water.
5. Stain with Schiff's reagent for 10 minutes.
6. Rinse in distilled water and wash well in tap water.
7. Counterstain with Mayer's hematoxylin or Carazzi's hematoxylin.
8. Blue in tap water, rinse in distilled water then mount sections in glycerine jelly.

Results

Gangliosides (in Tay-Sachs' disease and GM_1 gangliosidosis)	red
Nuclei	blue

Note

A chloroform methanol-extracted section should be used for comparison to exclude interference from non-lipid sialomucins.

Proteins and nucleic acids

Proteins and nucleic acids are major cell and tissue constituents. Proteins are highly organized complex macromolecules which are made up of 20 common amino acids linked together by peptide bonds. They occur in cells and tissues as simple and conjugated proteins. Simple proteins are made up of amino acids only, e.g. albumins, globulins, fibrous structure proteins and enzymes.

Phenyl groups

These can be demonstrated by a modification of the well-known biochemical test, the Millon reaction (Baker, 1956). A red or pinkish color develops at the site of tyrosine-containing proteins when the section is treated with a hot mercuric sulfate-sulfuric acid-sodium nitrite mixture. Tyrosine is the only amino acid which contains the hydroxyphenyl group in a form which can be demonstrated histochemically. The method can therefore be regarded as specific for tyrosine, but since tyrosine is an almost invariable constituent of all tissue proteins, the Millon reaction is a suitable general protein method. The color reaction of the Millon reaction is rarely strong and there may be difficulty encountered in keeping the sections on the slide. A less well-known, but more reliable, technique is the diazotization-coupling method (Glenner & Lillie, 1959).

Millon reaction for tyrosine (Baker, 1956)

Fixation

Neutral buffered formalin; formaldehyde vapor (for freeze-dried tissue).

Sections

Paraffin wax, fixed cryostat, freeze-dried or celloidin.

Solutions

Solution a

10 g of mercuric sulfate is added to a mixture of 90 ml distilled water and 10 ml of concentrated sulfuric acid, and dissolved by heating. After cooling to room temperature, 100 ml of distilled water is added.

Solution b

250 mg of sodium nitrite is dissolved in 10 ml of distilled water.

Staining solution

5 ml of Solution **b** is added to 50 ml of Solution **a**.

Method

1. Take sections to water.
2. Immerse sections in staining solution in a small beaker and gently bring to boil; simmer for 2 minutes.
3. Allow to cool to room temperature.
4. Wash in three changes of distilled water for 2 minutes each.
5. Dehydrate through alcohols, clear in xylene, and mount.

Result

Tyrosine-containing proteins	red or pink

Notes

a. A suitable positive control tissue is pancreas.
b. Mercury-containing reagents must be disposed of according to the law.
c. Caution should be used when diluting sulfuric acid with water.

Disulfide linkage

Performic acid-alcian blue method (Adams & Sloper, 1955)

Fixation

Neutral buffered formalin; formaldehyde vapor (for freeze-dried tissue).

Sections

Paraffin, freeze-dried, frozen cryostat sections.

Solutions

Performic acid solution

Concentrated formic acid	40 ml
30% hydrogen peroxide	4 ml
Concentrated sulfuric acid	0.5 ml

Alcian blue solution

Alcian blue	1 g
Concentrated sulfuric acid	2.7 ml
Distilled water	47.2 ml

Method

1. Take sections to water; blot to remove surplus water.
2. Immerse sections in performic acid solution (see Note a) for 5 minutes.
3. Wash well in tap water (see Note b) for 10 minutes.
4. Dry in 60°C oven until just dry.
5. Rinse in tap water.
6. Stain in Alcian blue solution at room temperature for 1 hour.
7. Wash in running tap water.
8. Counterstain (e.g. neutral red) if required.
9. Wash in tap water.
10. Dehydrate through alcohols, clear in xylene and mount.

Result

Disulfides	blue

The intensity of the blue color will depend on the amount of disulfide present.

Note

a. The performic acid solution should be prepared fresh and allowed to stand for 1 hour before use.
b. The section should be washed adequately but carefully; it may lift off if the washing is too vigorous following treatment in performic acid. The drying stage reduces the risk of section loss.

Indole groups

These can be demonstrated by the histochemical reaction of the amino acids tryptamine and tryptophan. The most reliable method is the DMAB-nitrite method of Adams (1957). The best results (i.e. most intense coloration and most precise localization) are obtained with freeze-dried sections, but satisfactory results are obtainable with paraffin sections. The principle of the method is that tryptophan reacts with DMAB (*p*-dimethylaminobenzaldehyde) to produce a substance known as β-carboline, which is then oxidized by the nitrite solution to produce a deep blue pigment.

DMAB-nitrite method for tryptophan (Adams, 1957)

Fixation

Neutral buffered formalin; formaldehyde vapor (for freeze-dried tissue).

Sections

Paraffin wax, freeze-dried, frozen cryostat sections.

Solutions

DMAB solution

5 g of *p*-dimethylaminobenzaldehyde is dissolved in 100 ml of concentrated hydrochloric acid.

Nitrite solution

1 g of sodium nitrite is dissolved in 100 ml of concentrated hydrochloric acid.

Method

1. Take sections to absolute ethanol.
2. Celloidinize in 0.5% celloidin.
3. Place sections in DMAB solution for 1 minute.
4. Transfer sections to nitrite solution for 1–2 minutes.
5. Wash gently in tap water for 30 seconds.
6. Rinse in acid alcohol for 15 seconds.
7. Wash in water and optionally counterstain in 1% aqueous neutral red for 5 minutes.
8. Dehydrate through ethanols, clear in xylene and mount.

Results

Tryptophan	dark blue
Nuclei	red

Notes

a. Pancreas is an excellent positive control tissue.
b. This is one of the most satisfactory and rewarding of amino acid histochemical methods.
c. The reagents give off toxic fumes and should be prepared and if possible, used in a fume hood.

Nucleic acids

Nucleoproteins are combinations of basic proteins (protamines and histones) and nucleic acids. The two nucleic acids are deoxyribonucleic acid (DNA), which is mainly found in the nucleus of the cell, and ribonucleic acid (RNA), which is located in the cytoplasm of cells, mainly in the ribosomes. Both the DNA and RNA molecules consist of alternate sugar and phosphate groups with a nitrogenous base (either a purine or pyrimidine) being attached to each sugar group. The sugar in DNA is the 5-carbon sugar deoxyribose; in RNA it is ribose. Histochemical techniques for the demonstration of nucleic acids in tissue are based on all their constituents. DNA and RNA can be localized in cells by the affinity of their negatively charged phosphate ester groups for almost any basic dye, particularly hematoxylin or methyl green and pyronin (Spicer, 1987). RNA is usually only evident in cells whose cytoplasm is particularly rich in this nucleic acid e.g. plasma cells and serous acinar cells. Hematoxylin is by no means specific for DNA and RNA, and it will also stain glycosaminoglycans and other anionic complexes.

Demonstration of nucleic acids

Fixation

In general terms, the nucleic acids are best preserved in alcoholic and acidic fixatives, a good example being Carnoy's fluid which contains both alcohol and glacial acetic acid. Formalin has only a limited reaction with DNA and RNA, but for routine work gives acceptable results. Low (4°C) temperature fixation in neutral buffered formalin has been shown to prevent DNA degradation by cell nucleases, which is of some importance when carrying out molecular biology studies (Tokuda et al., 1990).

Basophilia

DNA and RNA both stain strongly with most cationic dyes. Selectivity of staining can be achieved by using, for example, methylene blue at a pH range of 3.0–4.0. A distinction has been drawn between the link formed by simple cationic dyes such as neutral red and methylene blue and nucleic acid, and the nuclear stain formed by metal complex dyes such as alum hematoxylin (Marshall & Horobin, 1973). The former type of staining can be markedly reduced by prior treatment with acids, and is considered to be largely coulombic (electrostatic) in nature. Alum hematoxylin staining of cellular nuclei is much less affected by prior acid treatment. This part acid-fast effect is attributed to the metal-dye complex also forming non-electrostatic bonding interactions, such as hydrophobic bonding and van der Waals' attraction forces, with nucleic acids. Interestingly, whilst nucleic

acids exhibit strong basophilia, they do not usually exhibit metachromasia with the standard metachromatic dyes such as toluidine blue or azure A.

Deoxyribonucleic acid (DNA)

The typical demonstration of DNA is ether by the Feulgen technique, which will demonstrate the sugar deoxyribose, or the methyl green-pyronin technique in which the phosphates combine with the basic dye methyl green at an acid pH. DNA can also be demonstrated by fluorescent methods using acridine orange, although the reliability of this type of method is less than the previous methods. Both DNA and RNA can be demonstrated by the gallocyanin chrome alum method; the method does not separate the two nucleic acids and suitable extraction techniques must be used. The definitive, most sensitive technique for identifying DNA is that of in situ hybridization.

Feulgen reaction

The method of Feulgen and Rossenbeck (1924) is the standard technique for demonstrating DNA. Mild acid hydrolysis, employing 1 M hydrochloric acid at 60°C, is used to break the purine-deoxyribose bond; the resulting 'exposed' aldehydes are then demonstrated by the use of Schiff's reagent. The hydrolysis is the critical part of the method; an increasingly stronger reaction is obtained as the hydrolysis time is increased until the optimum is reached. Beyond this, the reaction becomes weaker, and if the hydrolysis is continued the reaction may fail completely. An important consideration in selecting the correct hydrolysis time is the fixative used. Bouin's fixative is not suitable as it causes over-hydrolysis of the nucleic acid during fixation. Bauer (1932) discussed the times of hydrolysis for various fixatives; some of these are reproduced in the sixth edition.

Feulgen nuclear reaction for DNA (Feulgen & Rossenbeck, 1924)

Fixation

Not critical but not Bouin's.

Solutions

1 M hydrochloric acid

Hydrochloric acid (conc.)	8.5 ml
Distilled water	91.5 ml

Schiff's reagent (see page 183)

Bisulfite solution

10% potassium metabisulfite	5 ml
1 M hydrochloric acid	5 ml
Distilled water	90 ml

Method

1. Bring all sections to water.
2. Rinse sections in 1 M HCl at room temperature.
3. Place sections in 1 M HCl at 60°C.
4. Rinse in 1 M HCl at room temperature for 1 minute.
5. Transfer sections to Schiff's reagent for 45 minutes.
6. Rinse sections in bisulfite solution for 2 minutes.
7. Repeat wash in bisulfite solution for 2 minutes.
8. Repeat wash in bisulfite solution for 2 minutes.
9. Rinse well in distilled water.
10. Counterstain if required in 1% light green for 2 minutes.
11. Wash in water.
12. Dehydrate through alcohols to xylene and mount.

Results

DNA	red-purple
Cytoplasm	green

Ribonucleic acid (RNA)

The method of choice for demonstrating RNA is the methyl green-pyronin technique. RNA can also be demonstrated along with DNA by acridine orange and by the gallocyanin-chrome alum technique, given suitable extraction procedures.

Methyl green-pyronin

This will demonstrate both DNA and RNA. Methyl green is an impure dye containing methyl violet. By removing the methyl with chloroform, the pure methyl green (when used at a slightly acid pH) appears to be specific for DNA.

The rationale of the technique is that both dyes are cationic when used in combination: methyl green binds preferentially, and specifically to DNA, leaving the pyronin to bind to RNA. The methyl green-specific reactivity is attributed to the spatial

alignment of the NH_2 groups of the dye to phosphate radicals on the DNA double helix. Pyronin staining, on the other hand, does not show this spatial affinity and any negatively charged tissue constituent will stain red. In practice, this means that as well as RNA, acid mucins present in epithelium as well as cartilage will stain.

Methyl green-pyronin method (Pappenheim, 1899; Unna, 1902 from Bancroft & Cook, 1994)

Fixation

Carnoy preferred, but formalin acceptable.

Solution

Methyl green pyronin Y

2% methyl green in distilled water (chloroform washed)	9 ml
2% pyronin Y in distilled water	4 ml
Acetate buffer, pH 4.8	23 ml
Glycerol	14 ml

Mix well before use.

Method

1. Take sections to water.
2. Rinse in acetate buffer, pH 4.8.
3. Place in methyl green pyronin Y solution for 25 minutes.
4. Rinse in buffer.
5. Blot dry.
6. Rinse in 93% ethanol, then in absolute ethanol.
7. Rinse in xylene and mount.

Results

DNA	green-blue
RNA	red

Digestion methods for nucleic acids

Specific enzymes can be used to digest DNA and RNA in tissue sections. Pure deoxyribonuclease will remove DNA, while ribonuclease will digest RNA. In a pure state the enzymes will not affect the other nucleic acid.

Enzyme extraction of DNA (Brachet, 1940)

Fixation

Potassium dichromate will inhibit digestion and should be avoided.

Digestion solution

Deoxyribonuclease	10 mg
0.2 M Tris buffer, pH 7.6	10 ml
Distilled water	50 ml

Method

1. Bring both test and control sections to water.
2. Place test section in extraction solution, control in Tris buffer pH 7.6, incubate both test and control section at 37°C for 4 hours.
3. Wash in running tap water.
4. Stain both sections by the Feulgen method.

Results

Test section	DNA negative
Control section	DNA red

Enzyme extraction of RNA (Brachet, 1940)

Fixation

Potassium dichromate and mercuric chloride should be avoided as digestion is inhibited.

Digestion solution

Ribonuclease	8 mg
Distilled water	10 ml

Method

1. Bring both test and control slides to water.
2. Place test slide in ribonuclease solution, and control slide in distilled water, incubate both test and control section at 37°C for 1 hour.
3. Wash in distilled water.
4. Apply methyl green-pyronin method.

Results

Test slide	RNA negative, DNA green
Control slide	RNA red, DNA green

Enzyme histochemistry

This subject has been largely replaced by immunohistochemistry. Below are some of the classical methods which are of diagnostic value.

Fixation for enzyme histochemistry

Enzymes are labile and their preservation is important. The mitochondria, in which many of the oxidative enzymes are located, are rapidly damaged

when the blood supply is cut. Freezing and subsequent thawing of blocks or sections damages organelles such as lysosomes which contain many of the hydrolytic enzymes. Hydrolytic enzymes show considerable diffusion if demonstrated on frozen, unfixed sections. Tissues to be used for the demonstration of the majority of hydrolytic enzymes may be subjected to controlled fixation, which, whilst decreasing the amount of demonstrable enzyme within the section, does allow for a considerably sharper localization of the remaining enzyme activity. Fixation will destroy many of the oxidative enzymes; there are a few exceptions to this, and these are indicated in the relevant enzyme methods. For the demonstration of hydrolytic enzymes, histochemical techniques are usually applied to sections which have been prefixed in cold (4°C) formal calcium.

Smears

In enzyme histochemistry, the use of smears for cytochemical identification and evaluation of cells is used. Such smears, whose preparation may be achieved in various ways, include blood, bone marrow and tissue cell suspensions. Three of the most useful enzymes are non-specific esterase, acid phosphatase and chloroacetate esterase. It is usual to fix smears before histochemical staining to preserve cell structure and enzyme localization.

Enzyme types

Oxidoreductases

- **Oxidases:** catalyze oxidation of a substrate in the presence of oxygen.
- **Peroxidases:** catalyze oxidation of a substrate by removing hydrogen, which combines with hydrogen peroxide.
- **Dehydrogenases:** catalyze oxidation of a substrate by removal of hydrogen.
- **Diaphorases:** catalyze oxidation of NADH and NADPH by removal of hydrogen.

Transferases

These catalyze the transfer of the radicals of two compounds without the loss or uptake of water.

Hydrolases

These catalyze the introduction of water or its elements into specific substrate bonds, although in some instances water may be removed. These enzymes include:

- Phosphatases (acid, alkaline and specific)
- Esterases
- Lipases
- Glycosidases
- Peptidases
- Pyrophosphatases.

Diagnostic applications

The two most common uses of enzyme histochemistry in surgical histopathology laboratories are:

- Skeletal muscle biopsy (see Chapter 18).
- Colonic biopsy in cases of suspected Hirschsprung's disease.

Colonic biopsy in cases of suspected Hirschsprung's disease

In the normal colon and rectum there are ganglion cells in both the submucosa and the so-called myenteric plexus between the circular and longitudinal muscle of the outer bowel wall. These ganglia, and their associated nerves, are responsible for colonic motility.

In Hirschsprung's disease in children, a variable segment of the rectum and colon is devoid of ganglion cells ('aganglionic segment'). In the affected segment peristalsis is impossible and the large bowel becomes obstructed. The diagnosis may be suspected clinically and radiologically but requires histological confirmation, usually by the examination of one or more suction biopsy specimens of rectal mucosa and submucosa with the aid of the enzyme histochemical method, acetylcholinesterase.

Acetylcholinesterase (from Filipe & Lake, 1983)

Preparation of tissue

Cryostat sections of snap-frozen tissue cut at 10 µm are air-dried and fixed for 30 seconds in 4% formaldehyde in 0.1 M calcium acetate (formal calcium).

Frozen sections of formal calcium-gum sucrose treated blocks of tissue.

Incubation medium

Acetylthiocholine iodide	5 mg
0.1 M acetate buffer, pH 6.0	6.5 ml
0.1 M sodium citrate	0.5 ml
30 mM copper sulfate	1 ml
Distilled water	1 ml
4 mM iso-octamethyl pyrophosphoramide (iso OMPA)	0.2 ml

Add 1.0 ml 5 mM potassium ferricyanide just before use.

Method

1. Rinse the fixed sections for 10 seconds in tap water.
2. Incubate at 37°C for 1 hour in the incubation medium.
3. Wash briefly in tap water.
4. Treat with 0.05% *p*-phenylene diamine dihydrochoride in 0.05 M phosphate buffer, pH 6.8 for 45 minutes at room temperature.
5. Wash in tap water.
6. Treat with 1% osmium tetroxide for 10 minutes at room temperature.
7. Wash well in tap water, counterstain lightly (10 seconds) in Carrazzi hematoxylin (or Mayer's hemalum), wash, dehydrate, clear and mount in DPX.

Results

Nerve fibers and cells containing acetylcholinesterase are stained dark brown to black.

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Appendix II

Tissue microarray

This is an abridged version of this topic in Chapter 6 of the 7th edition of this text

John D. Bancroft

Introduction

Tissue microarray (TMA) is a method used to evaluate numerous samples of tissue in a short time. First introduced by Battifora (1986) and further developed by Kononen et al. (1998) it is used to examine several histological sections at the same time by arraying them in a single paraffin block. TMA uses multiple tissue samples arranged in a single paraffin block using precision tools to prepare the recipient block.

Histological techniques play an important role in molecular biology and TMA has become a useful diagnostic tool, it conserves tissue samples saving time for both research and clinical work. TMA has applications in clinical pathology serving as quality control for new antibodies. The production of antibodies is an expensive and lengthy process, and TMA is a unique tool which aids the streamlining of the cumbersome validation and quality control of archival tissue as well as daily immunohistochemistry (IHC) controls.

TMA enables the study and evaluation of many diseases. A hollow needle is used to take numerous tissue core samples from specific areas of a pre-existing block of tissue and then these are placed in a single array block. Sections are taken from this block, the exact number depending on the size of the cores and the experience of the technologist, ultimately producing a single slide containing hundreds of tissue cores for review. The technique can be used for a wide range of staining procedures, including IHC, in situ hybridization (ISH), fluorescent in situ hybridization (FISH), special stain control samples and quality control sections for H&E. Only a small amount of reagent is used to analyze each slide, making TMA cost-effective, particularly with IHC and ISH techniques. TMAs have been widely used in IHC for quality control and assurance. They may demonstrate the antibody thresholds on a single slide which optimizes where the high and low signal intensities are seen.

Types of tissue microarrays

Prevalence TMAs are assembled from tumor samples of one or several types without attached clinical and pathological information. They are used to determine the prevalence of a given alteration in a specific area of interest in a tumor.

Progression TMAs contain samples of different stages of one tumor type and are used to discover associations between tumor genotype and phenotype. For example, a breast cancer progression TMA could contain samples of normal breast from patients with and without a history of breast cancer, different non-neoplastic breast diseases, ductal and lobular carcinoma in situ, invasive cancer of all stages, grades and histological subtypes, as well as metastases and recurrences after initially successful treatment.

Prognosis TMAs contain samples from tumors available with clinical follow-up data and represent a fast and reliable method for the evaluation of clinical importance of newly detected disease-related genes. Validation studies using prognosis TMAs can establish the associations between molecular findings and clinical outcomes.

Experimental TMAs are constructed from cell lines or samples from TMA archives for testing new antibodies and looking for gene targets.

Designing the grid

The design of the grid varies, depending on the purpose of the array, and needs considerable thought before tissue transfer occurs. The pathologist and technologist determine the guidelines according to the purpose and utilization of each specific laboratory. An array uses a series of 50 or more samples, set into one or several blocks. It is important to plan and record in advance how many samples will be arrayed, and to create a map or grid sheet. A large number of samples (high density) can be arrayed in a 37 × 24 × 5 mm block and a smaller number of samples (low density) in a 24 × 24 × 5 mm block.

Normal tissue controls and control cell lines are placed in columns between the tumors and normal tissue can be sited asymmetrically at one end of the block. Placing a notch at the end of the cassette block helps to confirm later that the orientation of the block is correct. Archived blocks can be used as a source of control tissue without destructive sampling. Making the tissue array is a multistep project. Selecting the slides, collecting the blocks and designing the grid consume the time, rather than the array process itself. Standardizing the construction of the grid makes it easier to follow, but it can still take several weeks before the array process begins. The organization of the blocks and slides is critical throughout the TMA process.

Fixation and processing of tissues and controls (see Chapters 4 and 6)

Preparation of the donor block

The file slides and blocks are reviewed to determine which blocks will be arrayed. The area of interest to be sampled is usually marked by circling with a pilot pen or permanent fine point marker, although some pathologists prefer to mark the blocks rather than the slides. Once the slides are reviewed and marked, the block is usually matched to the corresponding glass slide. It is important that the block is marked in the same area of interest as the marked slide. Donor blocks must be at least 1 mm thick to be suitable for array construction; if a marked area is less than 1 mm thick, two cores from this site are stacked on top of each other.

When marking the slides and blocks, the following colors can be used as indicators:

- Red – cancer
- Green – normal
- Black – pre-invasive.

It is important to keep the blocks and slides together. A filing system where a sectioned H&E case study slide is filed behind the archived control block is ideal.

Needle sizes

The size of the punch is critical in planning the TMA. There are four different sizes of punches; 0.6, 1.0, 1.5 and 2.0 mm. The 1.0 or 1.5 mm needles are recommended for general use, but the 0.6 mm needles can be used for 200 or more samples in blocks. The spacing between the cores must be planned carefully; spaces of 0.1 mm are ideal.

Database for tissue microarray analysis (Shaknovich et al., 2003)

The first step in the construction of a TMA is the selection of cases from a database and creation of a template or spreadsheet which identifies the position for each case and controls in the TMA block. During the viewing and photographing of a tested slide, the cores are referred to by their position, and each case in the database can be identified by the unique position of each core on the template.

Later, after the sections are stained, one image per core is taken and saved as a compressed file, then logged as its own position identifier. Image acquisition often takes less than one minute, including field selection, manual focusing and identification. It is sensible to save a set of images for each stain in an identified folder. Several images from different stains can be viewed on the screen, scored and the data entered manually onto the spreadsheet adjacent to the image cell. This method allows examination, consideration and scoring of multiple

cores and multiple stains of the same case with ease, allowing flexibility which is impossible at the microscope – where it requires changing slides, stains, light sources and identifying the correct area of tissue. Co-investigators can check scoring, and images can be printed and shared over an electronic network for large trials and also as an educational tool.

The automated arrayer is easy to use and includes a specimen tracking software system. The instrument marks, edits and saves punch coordinates using an on-screen display and software tools.

Arrayers

A manual arrayer relies on the designed map or grid sheet and direct visual identification and manual selection of the punching by the technologist, after the pathologist has marked the areas of interest on the slide. The number of cores punched per hour depends on the experience of the worker, but averages 30–70 cores. To prepare microarray blocks for special stain controls, or QC controls for H&E staining, an inexpensive pen extractor is suitable.

The portable quick ray arrayer is easy to handle and economical as well as portable. Its applications are the same as above but the needles are replaced by tips 1, 2, 3 or 5 mm in diameter. The recipient block is pre-prepared and sectionable with cores fitting easily into the holes. The block is then placed into an embedding mold with its cut surface face down and molten paraffin wax added to fill the mold before being allowed to solidify. The TMA is then ready to section.

Preparation of the recipient array block

A blank paraffin wax block is prepared and used as the recipient for the tissue samples. It is best to use soft paraffin wax and make sure there are no holes in the block caused by air bubbles. The number of specimens per array depends on the size of the punches and the desired array density. The 1.0 mm needle is favored as it gives a desirable core and leaves little distortion in the donor block.

To ensure the alignment of the punches, first move the recipient punch into position and make a mark in the paraffin wax. The same task is performed for the donor punch. Moving the needles uses the X or Y micrometer adjustment controls. The position of the punches over the block can be made by gently pushing down on them until a mark is made in the paraffin wax. One continues to make adjustments with the micrometer knobs until the desired position is attained.

The empty recipient block is placed in the holder and the attachment screws are tightened to keep this block from slipping. The recipient block is placed with its notched edge to the left of the block holder. Making a hole in the first position begins the array process. The smaller needle is used to create the hole. First the depth stop is adjusted and its nut is tightened to stop the needle at the correct depth. The needle is pushed downwards by hand, and the depth stop limits this motion. The handle in the needle is used to rotate the needle. The downward pushing pressure is then relieved and springs will pull the needle upwards. The stylet is used to empty the needle but this must not be removed from the needles during the array process. The donor block bridge is placed over the recipient block and the turret is moved to switch the larger needle into a vertical sampling position. The donor block is moved under the sampling needle and the larger needle is used to retrieve the sample. The needle is pushed downwards to retrieve the sample. The depth stop does not block the needle motion in the donor block and care must be taken to prevent the needle from entering too deep. It is best to have three punches from the same site, making the tissue sample well represented for the evaluation of prognostic markers. Use of a four block indexer allows four replicate blocks to be made at the same time. The donor core can be cut if it is too tall to fit in the recipient punch by ejecting the core with the stylus and placing it on a clean flat surface. A clean razor blade is used to cut the core to the desired length and it is then placed into the recipient block using a pair of forceps. The ability to study archival tissue specimens is important and cores can be stored in labeled Eppendorf tubes for use in future arrays.

Smoothing and sectioning

The array block must be smooth and level before sectioning. The easiest way to do this is to heat a clean microscopic slide to around 70–80°C and touch it to the array block surface. The surface of the block will begin to melt. Move the slide in a circular motion and place the slide and block in the refrigerator or freezer.

Microtomy

The water bath is set at 37°C. Gently face off the array block on a dedicated microtome and cut a hundred or more sections at 4–5 μm. One should place the sections on positively charged slides in the same orientation. It is advised to stain one slide for H&E and place the remaining unstained slides in a box for storage at –20°C.

Sections can be cut a day or two before they are stained, but to avoid contamination they are stored in slide boxes. Sectioned TMA blocks should be dipped in paraffin wax to seal the surface to avoid loss of antigens. Note that excessive soaking or freezing can cause the tissue to swell and keep the array block from ribboning well.

The tissue block can be cored several times with minimal distortion and on further sectioning of the whole block it is still possible in the majority of cases to make a diagnosis.

Troubleshooting and tips

- Core does not come out of the punch easily, the punch tip is bent or distorted: change the punch.
- Tissue core was pushed too deep: remove the sample with the small punch and replace with a new sample in the same position.
- Insufficient spacing of core: causes minor cracks or stress on the core when sectioning.
- Thinning of TMA cores in block: this is a result of repeated sectioning of the block, where cores are uneven.
- Loss of tissue on water bath: may be due to folds, wrinkles or mishandling of the ribbon.
- Re-facing block: if additional sections are required after the block has been filed it is important for continuity to reposition the block as close as possible to its original position on the microtome; hence the importance of using a dedicated microtome.
- Re-facing angle: incautious consideration shortens the life of the tissue microarray block, which is called thinning. It is important to make sure the cassette is completely flat on top of the mold.

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Applications of immunohistochemistry

Appendix III

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Introduction

The histological diagnosis is often clear from light microscopic examination of tinctorial-stained slides. However, in cases with varying differential diagnoses, or where molecular subtleties will add to case interpretation, immunohistochemistry may be employed to reach a conclusion. In certain circumstances, immunohistology also has a role in predicting prognosis and potential response to therapy.

It is beyond the scope of this text to provide a comprehensive list of all possible applications and the hundreds of antibodies currently available. Other excellent texts devoted entirely to that task are available (Further reading: Taylor & Cote, 2006; Dabbs, 2010) and there are several internet-based resources (e.g. Immunohistochemistry vade mecum, Paul Bishop, see below). This appendix attempts to provide an overview of the ways in which immunohistochemistry is commonly employed in the diagnostic pathology laboratory, alongside the basic immunohistochemical panels in common usage.

The most frequent application of immunohistology is to consider the nature of neoplasms whose morphology overlaps a range of diagnostic categories. However, other applications include identification of infective agents e.g. CMV, HSV, *Helicobacter. pylori*, deposition of immune complexes in renal and skin disease, and typing of active cell populations, e.g. Bcl-2, Ki67.

Classification of neoplasia

A common application of immunohistochemical staining is the classification of neoplasms according to the type of cellular differentiation. Previously, histochemistry and electron microscopy were used to identify cytoplasmic features which may indicate differentiation toward a particular cell type. However, immunohistochemistry has largely superseded electron microscopy.

Anaplastic tumors

These are typically composed of relatively large, pleomorphic cells with highly atypical nuclei which do not resemble any particular normal tissue type, i.e. they lack differentiation. The list of differential diagnoses is therefore wide and may include neoplasms of epithelial, hematopoietic, melanocytic, mesenchymal, and in the central nervous system, glial origin. Expense and tissue may be spared by performing stains in a tiered fashion, where the first wave of tests is designed to place a neoplasm into one of the major diagnostic categories, and the second and subsequent tiers are used for subclassification. Using this approach, the following table shows a typical panel of stains which would be used in the first tier of assessment.

Anaplastic tumors					
	Broad spectrum cytokeratins (AE1/3, MNF116, Cam 5.2)	**Lymphoid marker (CD45)**	**Melanocytic markers (Melan A, S100, HMB45)**	**Mesenchymal marker (Vimentin)**	**CNS[1] marker (GFAP)[2]**
Epithelial origin/ carcinoma	+	–	–	Some are positive	–
Hematopoietic/lymphoid	–	+	–	–	–
Melanocytic	–	–	+	–	–
Mesenchymal/sarcoma	+/–	–	+/–	+	–
Central nervous system/ glial	–	–	–	+/–	+

[1]Central nervous system.
[2]Glial fibrillary acid protein.

Small round cell tumors

This group of poorly differentiated neoplasms consists of small, immature/somewhat fetal-type cells with relatively round densely stained nuclei and scant cytoplasm. A panel of antibodies which would be useful in distinguishing among these differential diagnostic possibilities is shown in the following table.

Small round cell tumors							
	CD99	**CD45**	**Cytokeratin**	**Desmin**	**Neural markers (e.g. CD56)**	**Muscle markers (e.g. MyoD1)**	**WT1**
DIFFERENTIAL DIAGNOSIS IN CHILDREN AND ADULTS							
Ewing's sarcoma/PNET[1]	+	–	–/+	–	+	–	–
Neuroblastoma	–	–	–	–	+	–	–
Desmoplastic small round cell tumor	+	–	+	+	+	–	+
Wilms' tumor	–/+ (focal)	–	–/+ (blastema)	–/+ (blastema)	–/+ (blastema)	–	+
Rhabdomyosarcoma	+	–	–/+	+	–	+	–
Lymphoma	+	+	–	–	–	–	–
DIFFERENTIAL DIAGNOSIS PREDOMINANTLY IN ADULTS							
Synovial sarcoma	+	–	+	–	+	–	–
Small cell carcinomas	–	–	+	–	+	–	–

[1]Primitive neuroectodermal tumor.

Adenocarcinoma of unknown origin

Some neoplasms can be identified by the presence of specific tumor markers, e.g. prostate-specific antigen (PSA) in prostate, and thyroglobulin in thyroid. However, many neoplasms have no unique markers and require a panel of antibodies for diagnosis (Oien & Dennis, 2012). One example is the application of a panel of immunohistochemistry to a metastatic

tumor with glandular differentiation but no previously diagnosed primary site, or 'adenocarcinoma of unknown origin'. The panel used in this situation will necessarily differ for males and for females, see following table (modified from Oien & Dennis, 2012).

Adenocarcinoma of unknown origin								
Primary site (%)	**PSA**	**TTF1**	**GCDFP15**	**CDX2 and/or CK20**	**CK7**	**ER**	**Mesothelin**	**CA 125**
Breast	–	–	+/–	–	+	+/–	–	–/+
Colon	–	–	–	+	–	–	–/+	–
Lung	–	+	–	–	+	–	–/+	–/+
Ovary, serous	–	–	–	–	+	+/–	+	+
Ovary, mucinous	–	–	–	–/+	–/+	–/+	–/+	–/+
Pancreas	–	–	–	–/+	+	–	+/–	+/–
Stomach	–	–	–	–/+	+/–	–	–/+	–
Prostate	+	–	–	–	–	–	–	–

Spindle cell neoplasms

These neoplasms are composed of thin, elongated cells typically arranged in bundles and may occur at virtually any site in the body, including subcutaneous tissue, deep soft tissues, viscera and nervous systems. Most are mesenchymal or neuroectodermal in type, but lack specific histology clues as to their nature. The differential diagnoses and relevant immunohistochemistry (WHO, 2013) are shown in the table below.

Malignant spindle cell neoplasms								
	CD34	**ASMA[1]**	**Desmin**	**S100**	**CK**	**CD99**	**DOG-1**	**C-KIT**
Synovial sarcoma	–	–	–	–/+	+	+	–	–/+
MPNST[2]	–	–	–	+/–	–	–	–	–
Leiomyosarcoma	–	+	+	–	–/+	–	–	–
Low grade myofibroblastic sarcoma	–	+	–	–	–	–	–	–
Fibrosarcoma	–	–/+	–	–	–	–	–	–
Spindle cell carcinoma	–	–	–	–	+	–	–	–
Malignant melanoma	–	–	–	+	–	–	–	+ (in situ component)
GIST[3] (if site appropriate)	+	–	–	–/+	–	–	+	+

See also Germ cell table (p.21).

[1]Alpha smooth muscle actin.

[2]Malignant peripheral nerve sheath tumor.

[3]Gastrointestinal stromal tumor.

Lymphoma

The subclassification of lymphomas often involves molecular tests alongside histology and immunohistology. Several more examples of specific staining combinations for particular neoplasms are given in the table below (Kaufmann et al., 1999; Pileri et al., 2002; WHO, 2008; Mitrovic et al., 2009).

Immunohistochemistry in lymphoma diagnosis	
HODGKIN'S LYMPHOMA	
Classic Hodgkin's lymphoma	CD30+, CD15+, MUM1+, CD45–, EMA–, PAX5 +
NLPHL[1]	CD20+, CD30–, CD15–, MUM1–, CD3–, CD57–, CD79a, PAX5 +, OCT 2+, BOB 1+
MATURE B-CELL NEOPLASMS	
SLL/CLL[2]	CD20+, CD79a+, CD5+, CD23+, CD10–, cyclin D1–
Follicular lymphoma	CD20+, CD79a+, CD10+/-, Bcl-2 +, Bcl-6+, CD23–, CD5–, cyclin D1–
Mantle cell lymphoma	CD20+, CD79a+, CD5+, cyclin D1+, CD23–/+, CD10–, BCL6-, BCL2 +
Marginal zone lymphoma	CD20+, CD79a+, Bcl-2+, CD43 +(50%), Bcl-6–, CD10–, CD5–, cyclin D1–, CD23–
DLBCL[3]	CD20+, CD79a+, CD10/+/–, Bcl-6+/–, Bcl-2+/–, CD30+/–, MUM1+/–, CD5–/+, CD2&3–, PAX5 +, OCT2+, BOB1 +
Burkitt lymphoma	CD20+, CD79a+, CD10+, Bcl-6+, Ki 67 +++ (100%)), TdT–, Bcl-2–/+, CD34–, CD2&3–

[1]Nodular lymphocyte predominant Hodgkin's lymphoma.
[2]Small lymphocytic lymphoma/chronic lymphocytic lymphoma.
[3]Diffuse large B-cell lymphoma.

Mesothelioma versus adenocarcinoma

Mesothelioma can present with a variety of microscopic phenotypes, the commonest diagnostic conundrum being versus adenocarcinoma, although spindle cell variants of mesothelioma can parallel other spindle cell neoplasia. Immunohistochemistry (Carella et al., 2001; Ordóñez, 2007; Hussain et al., 2009) can be applied to differentiate between these two neoplasms in both surgical and cytological specimens. If proven to be an adenocarcinoma, a second round of antibodies may then be used to identify the primary source.

Epithelial malignant mesothelioma versus lung adenocarcinoma		
	Mesothelioma	**Adenocarcinoma**
Mesothelin	Usually positive	Usually negative
Calretinin	Usually positive	Usually negative
Cytokeratin 5/6	Usually positive	Usually negative
Wilms' tumor-1 (WT-1) gene product	Usually positive	Usually negative
Thrombomodulin (CD 141)	Usually positive	Usually negative
D2-40	Usually positive	Usually negative
Podoplanin	Usually positive	Usually negative
AE1/3	Usually positive (perinuclear accentuation)	Usually positive (peripheral membrane accentuation)
BerEP4	Usually negative	Usually positive
CEA[1]	Usually negative	Usually positive
Claudin 4	Usually negative	Usually positive
MOC31	Usually negative	Usually positive
HBME1	Usually negative	Usually positive
TTF1	Usually negative	Usually positive

[1]Carcinoembryonic antigen.

Germ cell tumors

There can be significant morphological overlap between the specific types of germ cell tumor, particularly classic seminoma, embryonal carcinoma and yolk sac tumor. Application of an immunohistochemical panel may aid accurate diagnosis and tumor classification (see table below). However, combined forms may exist, complicating interpretation.

Germ cell tumors (excluding teratoma differentiated)

	ITGCN[5]	Classic seminoma	Embryonal carcinoma (MTU[6])	Yolk sac tumor	Choriocarcinoma (MTT[7])
CD117 (c-kit)	+	+	–	–	–
PLAP	+	+	+	+	–/+
CD30	–	–	+	–	–
Epithelial marker (e.g. CK7, EMA[1])	–	–	+	–	+
OCT4	+	+	+	–	–
AFP[2]	–	–	–	+	–
HCG[3]	–	–	–	–	+
HPL[4]	–	–	–	–	+

[1]Epithelial membrane antigen.
[2]Alpha fetoprotein.
[3]Human chorionic gonadotropin.
[4]Human placental lactogen.
[5]Intratubular germ cell neoplasia.
[6]Malignant teratoma undifferentiated.
[7]Malignant teratoma trophoblastic.

Renal tumors

The majority of renal tumors can be diagnosed on H&E-stained sections. In cases with morphological uncertainty, the panel in the following table may be useful (Truong & Shen, 2011).

Renal tumors

	Clear cell carcinoma	Papillary carcinoma	Chromophobe carcinoma	Oncocytoma
Vimentin	+	+	–	–
RCC	+	+	–	–
CD10	+	+	–	–
CK7	–	+	+	–
CK19	–	+	+	+
C-kit	–	–	+	+
PAX2	+	+	–	+

Reactive versus neoplastic proliferations

Many reactive proliferations result in masses ('tumors') which may clinically mimic true neoplasms. Immunohistochemistry may play a role in differentiating reactive processes from subtle manifestations of neoplasia, typically by demonstrating the expression of proteins which are not usually expressed at detectable levels in normal tissues (see Further reading: Fletcher, 2007). Several examples are given in the table below.

Reactive versus neoplastic proliferations			
Lymph node	Follicular hyperplasia	Bcl-2 negative in follicles	Ki67 higher in follicles
	Follicular lymphoma	Bcl-2 positive in follicles	Ki67 same as rest of node
Prostate	Benign glands	p63 positive basal myoepithelial layer	P504S negative glands
	Adenocarcinoma	No p63 positive basal layer	P504S positive glands
Bladder	Benign/reactive	CK20 positive only in umbrella cells	
	Carcinoma in situ	CK20 positive in full thickness of urothelium	
Brain	Reactive gliosis	p53 and Ki67 are mostly negative	
	Low-grade astrocytoma	p53 positive astrocyte nuclei Ki67 shows high proliferation fraction	

Prediction of prognosis and therapeutic response

Immunohistochemistry can be used to identify biological processes or altered proteins which are related to the prognosis or likely therapeutic response of a given tumor. For example, objective measurement of the proliferative activity by the Ki67 labeling index (MIB-1 antibody) is an important component of the histological grading system for endocrine tumors of the intestines.

Another application is the detection of small metastatic deposits which are difficult to see by routine histological examination ('micrometastases') in sentinel lymph nodes, commonly applied in cases of melanoma and breast carcinoma. The clinical and prognostic significance of identifying such micrometastases is still under investigation (Balch et al., 2009).

Several examples of the application of immunohistochemistry in the selection of patients who would benefit from targeted therapies are given in the following table.

Examples of immunohistology staining reactions are presented in the sixth edition of this book.

Cell surface receptors	
Neoplasm	**Cell surface receptor**
GIST[1]	C-kit
Breast carcinoma	HER2[2], ER[3], PR[4]
Carcinoma colon and lung	EGFR[5]

[1]Gastrointestinal stromal tumor.
[2]Human epidermal growth factor receptor 2.
[3]Estrogen receptor.
[4]Progesterone receptor.
[5]Epidermal growth factor receptor.

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Internet resource

Immunohistochemistry vade mecum, http://e-immunohistochemistry.info/

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Technical Appendices

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Appendix IV

Measurement units

Jennifer H. Stonard

Introduction

SI Units (Système International d'Unités, World Health Organization, 1977) are used throughout this text. Under ISO 15189 (2012) there are two standards relating to measurement and measurement units (ISO 15189 standards F3.3 and F3.4). To attain ISO 15189 accreditation, laboratories must ensure equipment used for critical measurements be calibrated to SI units and verified fit for purpose. In every test there is a degree of uncertainty but where possible this must be minimized.

Structure of SI units

The SI consists of two types, base and derived units. It also includes a series of prefixes by means of which decimal multiples and submultiples of units can be formed.

Base units

Seven units have been selected to serve as the basis of the system. The four units relevant to the topics included in this book are given below.

SI base units

Quantity	Name of unit	Symbol for unit
Length	Meter (metre)	m
Mass	Kilogram	kg
Amount of substance	Mole	M
Time	Second	s

Derived units

If a base unit is multiplied by itself or by combining two or more base units, a group of units known as SI derived units are produced, as below.

SI derived units

Quantity	Name of derived unit	Symbol for unit
Area	Square meter	m^2
Volume	Cubic meter	m^3
Substance concentration	Moles per cubic meter	mol/m^3

Volume

The unit of volume is the cubic meter (m^3) but the liter (litre) where 1 liter = 1000 cm^3 = 10^{-3} m^3 is allowed. Squared and cubed are expressed as numerical powers and not by abbreviations. The volume in histology and scientific laboratories is specified either as liters, or more frequently as milliliters (ml), see table below.

Non-SI units to be retained for general use

Quantity	Unit	Symbol for unit	Value in SI units
Time	Minute	min	60 s
	Hour	h	3,600 s
	Day	d	86,400 s
Volume	Liter	l	1 dm^3 = 10^{-3} m^3
Mass	Tonne	t	1,000 kg

Length

The basic unit of length is the meter/metre (m) and all other units of length are expressed as multiples or submultiples of the meter, see below.

Length		
Unit	**Abbreviation**	**Size**
Meter	m	
Millimeter	mm	10^{-3} m
Micrometer	µm	10^{-6} m
Nanometer	nm	10^{-9} m
Picometer	pm	10^{-12} m

Mass

The basic unit is the kilogram (kg) and the working unit is the gram (g). The multiples and submultiples of the gram are shown in the table below.

Mass		
Unit	**Abbreviation**	**Size**
Milligram	mg	10^{-3} g
Microgram	µg	10^{-6} g
Nanogram	ng	10^{-9} g
Picogram	pg	10^{-12} g

Temperature conversion

Several temperature scales are used, the most common of which are the Fahrenheit scale (°F) and the Celsius scale (°C). To convert from Celsius to Fahrenheit, multiply the Celsius temperature by 9/5 and add 32. Thus, the temperature of boiling water, 100°C, converts to (9/5) (100°C) + 32 = 180 + 32 = 212°F. Similarly, the freezing point of water is 0°C or 9/5 (0°) + 32 = 32°F. To convert from Fahrenheit, first subtract (32°F) and then multiply by 5/9. The 212°F boiling point converts to (212 – 32) × 5/9 = 180 (5/9) = 100°C. The following table provides some simple conversions.

Conversion of °C to °F	
°C	**°F**
–80	–112
–70	–94
–40	–40
–20	–4
–17.7	0
–10	14
0 (water freezes)	32
10	50
20	68
30	86
40 (hot day)	104
50	122
60	140
70	158
80	176
90	194
100 (water boils)	212

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Appendix V

Preparation of solutions

Danielle McCluskey

Introduction

Most solutions in histology are made using water as a solvent. The agent dissolved in water to make an aqueous solution is the solute. Solutions typically are made as volume to volume or weight to volume. Concentrated formaldehyde is a 40% solution of formaldehyde (CH_2O) in water. This represents the maximum solubility of the molecule (solute), in water (the solvent) and the resulting solution is a 40% w/v (weight-to-volume) solution.

The majority of histology laboratories now purchase commercially produced solutions in a prepared form. This is largely to reduce user exposure to a variety of hazardous chemicals and stains, thus minimizing any associated risk. Additionally, the use of commercial solutions is beneficial in maintaining quality, as they produce standardization of the staining.

Volume-to-volume solution

Making 10% formalin from a concentrated solution of formaldehyde (40% w/v) is an example of preparing a volume-to-volume solution. Add one part of concentrated formaldehyde to nine parts of water and this yields a 10% solution of formalin equivalent to a 4% solution of formaldehyde. Ten percent formalin is buffered to make 10% neutral buffered formalin, the most commonly used fixative in the USA and Europe.

For accuracy in preparing a solution, measure small volumes using volume-calibrated pipettes, if available. The accuracy of a dilute solution is based more upon the accuracy of the measurement of the solute than of the measurement of the solvent. Thus, if 1.1 ml of solute is added instead of 1 ml, the error is 10% in the concentration of a 1% solution. However, if 1 ml of solute is added to 99.1 ml instead of 99 ml of solvent, the error is less than 1%.

A stock solution is generally used to prevent having to measure small amounts of the solute and improves molecular stability. From a 1% stock solution, a 0.1% solution is prepared by adding 10 ml of 1% solution to 90 ml of water, and a 0.01% solution is prepared by adding 1 ml of 1% stock solution to 99 ml of water.

Use reagent grade chemicals and distilled/deionized water ideally, but if considering stock solutions then the initial state of hydration specified for the material being dissolved is relevant for the ultimate weight per volume solute when diluting the sample with more water.

In preparing a modified solution from the original solutions, the following equation is useful:

$$V1 \times C1 = V2 \times C2$$

where V = volume, C = concentration and the different solutions are 1 and 2

Examples of its use are as follows and see table below:

- Using 100 ml of a solution of 1% sodium hydroxide, prepare a 0.5% solution of sodium hydroxide:

$$100\ \text{ml} \times 1\% = X\ \text{ml} \times 0.5\%$$
$$X\ \text{ml} = 200\ \text{ml}$$

Therefore, add 100 ml of water to the 100 ml original solution to go from 100 ml of 1% to 200 ml of 0.5% sodium hydroxide.

- Using 50 ml of 5% potassium permanganate, prepare a 2% solution of potassium permanganate:

$$50 \text{ ml} \times 5\% = X \text{ ml} \times 2\%$$
$$X \text{ ml} = 125 \text{ ml}$$

Therefore, add 75 ml of water to the 50 ml original solution to go from 50 ml of 5% to 125 ml of 2% potassium permanganate.

Volume-to-volume solutions: preparation of 100 ml of a solution		
% aqueous solution	**ml of solute**	**ml of water**
1%	1 ml	99 ml
5%	5 ml	95 ml
10%	10 ml	90 ml
50%	50 ml	50 ml

Weight-to-volume solutions

Weight-to-volume solutions are typically used when a weight of a solute, typically a solid, is dissolved in an aqueous or other solvent. In such a preparation, assume the weight of 100 ml of water is 100 grams. To make a 1% solution of potassium permanganate, add 1 gram of potassium permanganate to 99 ml of water and make the final solution up to 100 ml. This usually requires a 100 ml volumetric flask. In histology, such accuracy is seldom required and a good approximation is to dissolve the solute in 100 ml of the solvent. Just like volume-to-volume solutions, do not try to measure small quantities of the solute (less than 1 gram). If a dilute solution is to be made, dilute a more concentrated solution, as shown below for solutions of 0.1% or less. As above, be careful to note the state of hydration of the chemical being used. The weight of the complexed water must be calculated and removed from the chemical weight.

To avoid measuring less than 1 g, change the preparation from weight-to-volume to a volume-to-volume solution, as shown in the following examples and table below:

- Prepare a 0.0025% solution of sodium chloride. (Note for a % weight-to-volume solution the molecular weight of NaCl is not needed.) Start by preparing a 2.5% solution or 2.5 g of NaCl in 100 ml of water. Add 1 ml of this 2.5% solution to 1000 ml of water. This would provide a 0.0025% solution without weighing or measuring small quantities.
- How much water do you add to 100 ml of 20% sodium chloride to obtain a 7.5% solution? Using the V1 × C1=V2 × C2 equation:

$$100 \text{ ml} \times 20\% = X \text{ ml} \times 7.5\%$$
$$X \text{ ml} = 2000/$$
$$7.5 = 267 \text{ ml}$$

You need to add 167 ml of water to the 100 ml of 20% solution to produce 267 ml of a 7.5% solution.

Preparation of weight-to-volume solutions		
% weight	**Solute**	**Solvent**
1	1 g	100 ml
2.5	2.5 g	100 ml
5	5 g	100 ml
7.5	7.5 g	100 ml
10	10 g	100 ml
0.1	10 ml of 1%	90 ml
0.01	1 ml of 1%	99 ml
0.001	1 ml of 0.1%	99 ml

Molar solutions (M)

These are based upon the molecular weight of the solute. The molecular weight is normally stated on the label of the container of the chemical. It can be looked up in the *Merck Index* or *CRC Handbook of Chemistry*. If water is bound to the solute, the amount of water typically bound at laboratory conditions must be considered in weighing the molecular weight of the solute. A 1 molar solution is the molecular weight in grams of the solute dissolved in 1 liter (1000 ml) of the solvent. A 1 molar solution of sodium chloride, molecular weight = 58.5 g, is prepared by dissolving 58.5 grams of sodium chloride in 1 liter of water. A 0.1 molar solution of sodium chloride is 5.85 grams dissolved in 1 liter. See table below.

Preparation of molar solutions		
Solution	**Weight of solute**	**Final volume of solution**
1 molar	Molecular weight in grams	1000 ml
0.1 molar	0.1 × molecular weight	1000 ml
0.01 molar	10 ml of 0.1 molar	100 ml (90 ml solvent)
0.001 molar	10 ml of 0.01 molar	100 ml (90 ml solvent)

Normal solutions (N)

The preparation of a normal solution is based upon dissolving an equivalent weight of an equivalent single positive ionic species. Sometimes this is called the equivalent (molecular) weight. For example, if only one positive ion is present in a molecule, such as sodium chloride (NaCl), then the equivalent weight is the same as the molecular weight and a 1 normal solution is the same as a 1 molar solution; thus, one molecular weight of sodium ions is present in the 1 N solution. By contrast, potassium sulfate (K_2SO_4) has two positive ions per molecule; thus, the equivalent weight is the molecular weight divided by 2. In the case of calcium chloride ($Ca(Cl)_2$), the calcium with a positive charge (valence) of 2 is equivalent to two positive ions, so the normal (equivalent) weight is half the molecular weight. Thus, the equivalent weight used to prepare normal solutions depends on the molecular weight and the ionic form of the molecule. The preparation of normal solutions is shown below.

Preparation of normal solutions		
Normality	**Species**	**Equivalent weight**
1 N	$X^{+1}Y^{-1}$	Molecular weight
1 N	$X^{+2}Y^{-2}$	½ the molecular weight
1 N	$X^{+3}(Y^{-1})_3$	⅓ the molecular weight
0.5 N	$X^{+1}Y^{-1}$	½ the molecular weight
0.5 N	$X^{+2}Y^{-2}$	¼ the molecular weight
0.1 N	Any species	1 part 1 N and 9 parts water
0.01 N	Any species	1 part 0.1 N and 9 parts water
0.001 N	Any species	1 part 0.1 N and 99 parts water

Preparation of useful solutions

IMPORTANT: Chemicals should not be used if not in their original container.

Acid alcohol

70% alcohol	99 ml
Concentrated hydrochloric acid	1 ml

Acid permanganate

0.5% aqueous potassium permanganate	50 ml
3% sulfuric acid	2.5 ml

Gram's iodine

Iodine	3 g
Potassium iodide	6 g
Distilled water	900 ml

Lugol's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	100 ml

Alcian blue solution at various pH

pH 0.2	1 g in 100 ml of 10% sulfuric acid
pH 0.5	1 g in 100 ml of 0.2 M hydrochloric acid
pH 1.0	1 g in 100 ml of 0.1 M hydrochloric acid
pH 2.5	1 g in 100 ml of 3% acetic acid
pH 3.2	1 g in 100 ml of 0.5% acetic acid

Magnesium chloride solution (for use with alcian blue at different electrolyte concentrations)

0.05 M	1.01 g in 100 ml of distilled water
0.06 M	1.22 g in 100 ml of distilled water
0.3 M	6.09 g in 100 ml of distilled water
0.5 M	10.15 g in 100 ml of distilled water
0.7 M	14.21 g in 100 ml of distilled water
0.9 M	18.27 g in 100 ml of distilled water

Saturated picric acid solution

The solubility of picric acid is 1.2 g/100 ml or 1.2%. Due to the hazards of dry picric acid, it is typically sold in a form containing 30–35% water.

Picric acid (hydrated)	1.6 g
Distilled water	100 ml

Note: Solutions of picric acid should not be allowed to evaporate, as dry picric acid is potentially explosive.

Scott's tap water

Potassium bicarbonate	2 g
Magnesium sulfate	20 g
Distilled water	1000 ml

Tris-HCl buffered saline, pH 7.6 for immunoperoxidase wash

Sodium chloride	8.1 g
Tris(hydroxymethyl)aminomethane	0.6 g
1 M HCl	3.8 ml
Distilled water	1000 ml

Appendix VI

Buffer solutions

Paul Samuel

Introduction

The pH of a solution is defined as the logarithm to base 10 of 1 divided by the concentration of the free hydrogen ions in solution (i.e. pH = $\log_{10} 1/[A^+]$ = $-\log_{10}[H^+]$). A neutral solution is defined as pH = $-\log_{10}[10^{-7}]$ = 7. The pH may greatly affect many chemical and immunohistochemical reactions, and consequently it is frequently important to minimize large changes in free hydrogen ion content, i.e. stabilize the pH.

Buffers are typically solutions in which the addition of small quantities of acids or bases causes little or no change in the pH of the solution. In other words, the solution 'buffers' against a change in pH. This is accomplished by solutions of inorganic and organic acids or bases plus salts, which together absorb free hydrogen or free hydroxyl ions to prevent major changes in pH. Several major buffer systems are used in histochemical and/or immunohistochemical staining. Buffer systems include citric acid, sodium citrate, acetic acid-sodium acetate and mixtures of sodium or potassium phosphates. One frequently used system is based on the use of Tris(hydroxylmethyl)aminomethane, often called 'Tris'. Tris buffer systems include Tris-maleic acid. Tris buffers are susceptible to temperature changes, so pH values specified at multiple temperatures are shown. The following buffer tables are the primary buffers referred to in this edition. For any buffers required and not listed here, the reader is referred to Pearse (1980) and Lillie and Fullmer (1976), or to suitable biochemical texts.

General notes regarding buffer solutions

The salts and acids used in the preparation of buffers should be of at least laboratory reagent grade. When preparing buffers, the molecular weight given on the reagent bottle should be checked, as many chemicals are available in a number of states of hydration.

Acetate buffer

Preparation of stock solutions

Stock A: 0.2 M acetic acid (MW 60.05)

1.2 ml of glacial acetic acid in 100 ml of distilled water.

Stock B: 0.2 M sodium acetate (MW 136)

1.64 g of sodium acetate trihydrate in 100 ml of distilled water.

Composition of buffer

x ml of **A** + *y* ml of **B** made up to 100 ml with distilled water

Acetate buffer

pH	*x* ml of A	*y* ml of B
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

Cacodylate buffer

Preparation of stock solutions

Stock A: 0.2 M sodium cacodylate (MW 214)

4.28 g of sodium cacodylate in 100 ml of distilled water.

Stock B: 0.2 M HCl (MW 36.46)

1.7 ml of hydrochloric acid in 100 ml of distilled water.

Composition of buffer

25 ml of **A** + *y* ml of **B** made up to 100 ml with distilled water.

Cacodylate buffer

pH	*y* ml of B
5.0	23.5
5.2	22.5
5.4	21.5
5.6	19.6
5.8	17.4
6.0	14.8
6.2	11.9
6.4	9.2
6.6	6.7
6.8	4.7
7.0	3.2
7.2	2.1
7.4	1.4

Phosphate buffer

Preparation of stock solutions

Stock A: 0.2 M sodium dihydrogen orthophosphate (MW 156)

3.12 g of sodium dihydrogen orthophosphate in 100 ml of distilled water.

Stock B: 0.2 M disodium hydrogen orthophosphate (MW 142)

2.83 g of disodium hydrogen orthophosphate in 100 ml of distilled water.

Composition of buffer

x ml of **A** + *y* ml of **B** made up to 100 ml with distilled water.

Phosphate buffer

pH	*x* ml of A	*y* ml of B
5.8	46.0	4.0
6.0	43.8	6.2
6.2	40.7	9.3
6.4	36.7	13.3
6.6	31.2	18.8
6.8	25.5	24.5
7.0	19.5	30.5
7.2	14.0	36.0
7.4	9.5	40.5
7.6	6.5	43.5
7.8	4.2	45.8
8.0	2.6	47.4

Phosphate-citrate buffer

Preparation of solutions

Stock A: 0.2 M disodium hydrogen orthophosphate (MW 142.0)

2.83 g of disodium hydrogen orthophosphate in 100 ml of distilled water.

Stock B: 0.1 M citric acid (MW 210.0)

2.1 g of citric acid in 100 ml of distilled water.

Composition of buffer

x ml of **A** + (100 – *x*) ml of **B**

Phosphate-citrate buffer

pH	*x* ml of A	ml of B
3.6	32.2	67.8
3.8	35.5	64.5
4.0	38.5	61.5
4.2	41.4	58.6
4.4	44.1	55.9
4.6	46.7	53.3
4.8	49.3	50.7
5.0	51.5	48.5
5.2	53.6	46.4
5.4	55.7	44.3
5.6	58.0	42.0
5.8	60.4	59.6
6.0	63.1	36.9
6.2	66.1	33.9
6.4	69.2	30.8
6.6	72.7	27.3
6.8	77.2	22.8
7.0	82.3	17.7
7.2	86.9	13.1
7.4	90.8	9.2
7.6	93.6	6.4
7.8	95.7	4.3

Tris-HCl buffer

Preparation of stock solutions

Stock A: 0.2 M Tris (MW 121.0)

2.42 g of Tris(hydroxymethyl)aminomethane in 100 ml of distilled water.

Stock B: 0.2 M HCl (MW 36.46)

1.7 ml of hydrochloric acid in 100 ml of distilled water ***(note: water should be added to acid, NOT vice versa).***

Composition of buffer

25 ml of **A** + *y* ml of **B** made up to 100 ml with distilled water.

Tris-HCl buffer

pH	*y* ml of B
7.2	22.1
7.4	20.7
7.6	19.2
7.8	16.3
8.0	13.4
8.2	11.0
8.4	8.3
8.6	6.1
8.8	4.2
9.0	2.5

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Solubility of some common reagents and dyes

Appendix VII

Stuart Inglut

The following table shows the solubility of useful reagents where the weight of solute, in the third column, dissolved in the volume of distilled water, in the fourth column, will produce 100 ml of a saturated solution at the temperature given in the second column. When considering the solubility of a reagent, there are three possible results:

1. If the solution has less solute than the maximum amount that it is able to dissolve (its solubility), it is a dilute solution.
2. If the amount of solute is exactly the same amount as its solubility, it is saturated.
3. If there is more solute than is able to be dissolved, the excess solute separates from the solution. If this separation process includes crystallization, it forms a precipitate. Precipitation lowers the concentration of the solute to the saturation in order to increase the stability of the solution.

Solubility of useful reagents

	Temperature (°C)	Weight of solute (g)	Volume of distilled water (ml)
Aluminum ammonium sulfate	25	13.00	92.0
Aluminum potassium sulfate	25	7.02	99.1
Aluminum sulfate	25	63.00	66.0
Ammonium molybdate	25	39.00	88.0
Ammonium nitrate	25	90.20	41.8
Ammonium oxalate	25	5.06	97.0
Calcium chloride	25	67.80	79.2
Chloral hydrate	25	120.00	31.0
Citric acid	25	88.60	42.7
Cobalt nitrate	18	78.20	79.1
Cupric sulfate	25	22.30	98.7
Dextrose	25	59.00	60.0
Ferric ammonium sulfate	16.5	22.40	94.3
Ferric chloride	25	131.10	48.3
Ferric nitrate	25	70.20	79.2

Continued

Solubility of useful reagents—cont'd			
	Temperature (°C)	**Weight of solute (g)**	**Volume of distilled water (ml)**
Glycine	25	21.70	86.8
L-Glutamic acid	25	0.86	99.15
Hydroquinone	20	6.78	94.4
Lead nitrate	25	53.60	91.0
Lithium carbonate	15	1.38	100.0
Magnesium chloride	25	79.00	47.5
Magnesium nitrate	25	58.60	80.5
Magnesium sulfate	25	72.00	58.5
Oxalic acid	25	10.30	94.2
Phenol crystals	20	6.14	94.5
Phosphomolybdic acid	25	135.00	46.0
Phosphotungstic acid	25	160.00	64.0
Potassium acetate	25	97.10	44.3
Potassium bicarbonate	25	31.60	87.5
Potassium bromide	25	56.00	82.0
Potassium carbonate	25	82.20	73.5
Potassium chloride	25	31.20	86.8
Potassium dichromate	25	14.20	95.0
Potassium ferricyanide	22	38.10	80.8
Potassium ferrocyanide	25	28.20	89.2
Potassium hydroxide	15	79.20	74.2
Potassium iodide	25	103.20	69.1
Potassium nitrate	25	33.40	86.0
Potassium permanganate	25	7.43	97.3
Resorcin	25	67.20	47.2
Silver nitrate	25	164.00	65.5
Sodium acetate	25	40.50	80.0
Sodium bicarbonate	15	8.80	97.6
Sodium carbonate	25	28.10	96.5
Sodium chloride	25	31.70	88.1
Disodium hydrogen orthophosphate	17	4.40	99.9
Sodium hydroxide	25	77.00	74.0
Sodium hypophosphite	16	72.40	66.6
Sodium iodate	25	9.21	98.5
Sodium nitrite	20	62.30	73.8

Solubility of useful reagents—cont'd			
	Temperature (°C)	**Weight of solute (g)**	**Volume of distilled water (ml)**
Sodium periodate	25	13.90	96.2
Sodium sulfate	25	28.50	95.5
Sodium sulfite	25	26.40	94.5
Sodium thiosulfate	25	93.00	46.0
Sucrose	25	90.00	43.0
Trichloroacetic acid	25	149.60	12.41

The following table shows the solubility of some commonly used dyes.

Solubility of some dyes				
			Approximate solubility (g/100 ml)	
Dye name	**Generic name**	**Color index no.***	**Water**	**Alcohol**
Acridine Orange	Basic Orange 14	46005	Sol	Sol
Alcian Blue 8GX	Ingrain Blue 1	74240	5	1.6
Alizarin Red S	Mordant Red 3	58005	7.5	0.15
Aniline Blue (H_2O Sol) (Sol blue 3M or 2R, water blue)	Acid Blue 22	42755	Sol	Slight
Auramine O	Basic Yellow 2	41000	0.7	4.5
Azophloxine	Acid Red 1	18050	3	Slight
Azure A (McNeal)	–	52005	Sol	Sol
Biebrich Scarlet	Acid Red 66	26905	Sol	0.05
Bismark Brown Y (Vesuvian Brown)	Basic Brown 1	21000	1.3	1.1
Carmine	Natural Red 4	75470	Sol	Slight
Carminic acid	Natural Red 4	75470	8.3	–
Chromotrope 2R	Acid Red 29	16570	19	0.15
Congo Red	Direct Red 28	22120	Sol	0.2
Cresyl Fast Violet	–	–	Sol	Slight
Crystal Ponceau 6R (brilliant Crystal Scarlet 6R, Ponceau 6R)	Acid Red 44	16250	3	0.5
Crystal Violet	Basic Violet 3	42555	1.7	13
Eosin Bluish (Eosin B, Erythrosin B)	Acid Red 51	45430	11	2

Continued

Solubility of some dyes—cont'd

Dye name	Generic name	Color index no.*	Approximate solubility (g/100 ml)	
			Water	Alcohol
Eosin Yellowish (H_2O & alcohol Sol, Eosin Y)	Acid Red 87	45380	44	2
Fast Garner GBC salt	Azoic Diazo component 4	37210	5	–
Fast Green FCF	Food Green 3	42053	16	0.35
Fast Red B salt	Azoic Diazo component 5	37125	20	–
Fast Red TR salt	Azoic Diazo component 11	37085	20	–
Fluorescein	Acid Yellow 73	45350	–	2.1
Fuchsin acid	Acid Violet 19	42685	20	0.25
Fuchsin basic	Basic Violet 14	42510	0.4	8
Fuchsin new	Basic Violet 2	42520	1.13	0.41
Gallocyanine	Mordant Blue 10	51030	Insol	Slight
Hematoxylin	Natural Black 1	75290	1.5	>30
Indigo carmine	Food Blue 1	73015	1.1	–
Janus Green B	–	11050	5.3	1.1
Light Green SF	Acid Green 5	42095	20	0.8
Luxol Fast Blue	Solvent Blue 38	–	V. Sol	Sol
Malachite Green	Basic Green 4	42000	7.60	7.52
Martius Yellow	Acid Yellow 24	10315	4.5	0.15
Metanil Yellow	Acid Yellow 36	13065	5.36	1.45
Methyl Blue	Acid Blue 93	42780	Sol	Slight
Methyl Green	Basic Blue 20	42585	Sol	Insol
Methyl Violet 2B	Basic Violet 1	42535	3	15
Methylene Blue	Basic Blue 9	52015	3.5	1.5
Neutral Red	Basic Red 5	50040	5.5	2.5
Nile Blue sulfate	Basic Blue 12	51180	1.0	1.0
Oil Red O	Solvent Red 27	26125	Insol	0.5
Orange G	Acid Orange 10	16230	10	0.2
Patent Blue	Acid Blue 1	42045	8.4	5.23
Phloxin	Acid Red 92	45410	50	9
Phosphine	Basic Orange 15	46045	Sol	Sol
Picric acid	–	10305	1.2	8
Ponceau 2R (Ponceau de xylidene)	Acid Red 26	16150	6	0.1

Solubility of some dyes—cont'd

Dye name	Generic name	Color index no.*	Approximate solubility (g/100 ml)	
			Water	Alcohol
Pyronin Y (Pyronin G)	–	45005	9	0.6
Rhodamine B	Basic Violet 10	45170	0.8	1.5
Safranin O	Basic Red 2	50240	5.5	3.5
Solochrome cyanine RS (Eriochrome cyanine R)	Mordant Blue 3	43820	Sol	Sol
Scarlet R (Sudan IV)	Solvent Red 24	26105	Insol	0.2
Sudan Black B	Solvent Black 3	26150	Insol	1.13
Tartrazine	Food Yellow 4	19140	11	0.1
Thioflavine T	Basic Yellow 1	49005	Sol	Sol
Thionin	–	52000	0.25	0.25
Toluidine Blue	Basic Blue 17	52040	3.8	0.5
Victoria Blue B	Basic Blue 26	44045	0.5	4

*From Colour Index International, third ed. Bradford, UK: Society of Dyers and Colourists and American Association of Textile Chemists and Colorists.
Sol = soluble, Insol = insoluble, V. Sol = very soluble.

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Mounting media and slide coatings

Appendix VIII

Ann Michelle Cull

Introduction

In order to provide the maximum degree of transparency to stained tissue sections, the refractive index of the mounting medium must approximate to that of dried protein, i.e. between 1.53 and 1.54. This is especially important for photographing slides. To visualize detail in unstained tissues, it may be desirable to employ a medium with lower or higher refractive index. The refractive index of a mounting medium may change on drying due to evaporation of solvents. Air bubbles should not be permitted to remain under coverslips since these tend to expand.

Mountants

Most pathology laboratories currently utilize commercial mounting media, usually non-aqueous. From the standpoint of safety and costs, this may be a laboratory's best approach to mounting sections.

Potassium acetate may be added to mountants, almost to the point of saturation, in order to reduce the 'bleeding' of cationic dyes, at the same time giving a pH of approximately 7.0.

Aqueous mounting media are required for some special stains, e.g. Oil Red O where using solvents would dissolve the fats being demonstrated. In direct immmunofluorescent stained slides the aqueous mountant should be fluorescence free. Few aqueous mountants have a refractive index higher than 1.5, most being in the range 1.4 to 1.45. Higher refractives are usually achieved by the use of high concentrations of sugars.

For other specialized mountants, see the appendices of the 6th edition of this text.

Apathy's mountant (modified by Lillie & Ashburn, 1943), refractive index 1.41

Gum Arabic crystals	50 g
Cane sugar	50 g
Distilled water	100 ml
Thymol	100 mg

Dissolve with moderate heating.

Apathy's mountant (Highman's modification, 1946), refractive index 1.436

Gum Arabic crystals	50 g
Cane sugar	50 g
Potassium acetate	50 g
Distilled water	100 ml

Gentle heat may be used to dissolve the solids. 0.05 g of thymol or merthiolate may be added as a preservative.

Combined coverslip and mountant

Several manufacturers supply a medium of a varnish-like nature which may be used to coat the section surface by dipping, pouring or spraying. This type of medium obviates the need for a coverslip.

For low-power microscopy, combined mountant and coverslip may prove quite satisfactory although little protection of the section to abrasion is given. High-power microscopy demands the use of optically flat slide surfaces with a coverslip of known thickness.

Adhesive slides

Slides may be chemically coated or charged to give them adhesive properties. Microscopic slides may be coated or charged for a variety of reasons:

1. To act as an adhesive to keep difficult tissue specimens attached e.g. fatty tissues such as breast; hard tissues such as bone, synovium and cartilage; or cellular preparations.
2. To aid in cellular attachment when cells are to be grown on slides or coverslips.
3. To make staining more consistent when staining small structures, e.g. cultured cells which spread across microscope slides.

Slides with adhesive properties are often bought from commercial suppliers. However, if necessary it is possible to create adhesive slides within the laboratory.

Silanized (APES) slides

These are prepared by cleaning the slides by washing, followed with a rinse in 95% ethanol. Approximately 4 ml of 3-aminopropyltriethoxy silane is added to 200 ml of acetone and slides are dipped for 30–60 seconds, followed by 60 seconds in agitated distilled water. The coated slides are then dried for 1 hour, and can be boxed for future use.

Polylysine (PLL) slides

These are prepared similarly to silanized slides. First wash the slides, followed by a rinse in 95% ethanol. Immerse the non-frosted portion of slides in a 5% solution of polylysine for more than 1 minute. Dry for over 1 hour. The slides can be boxed for future use.

Albumen-coated slides

Wash slides followed by a 95% ethanol wash. Make a 5% solution of crude egg albumen Grade II and immerse non-frosted portion of slides for over 1 minute. Permit to dry for 1 hour and then fix in a solution of 10% neutral buffered formalin for over 1 hour. Dry and box slides for future use.

References

Highman, B. (1946). Improved methods for demonstrating amyloid in paraffin sections. *Archives of Pathology*, *41*, 559–562.

Lillie, R. D., & Ashburn, L. L. (1943). A modification of Apathy's mounting medium. *Archives of Pathology*, *36*, 432.

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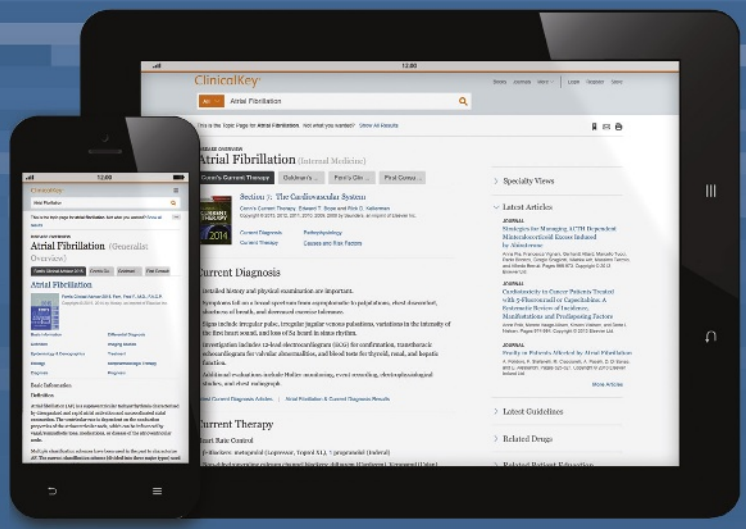

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