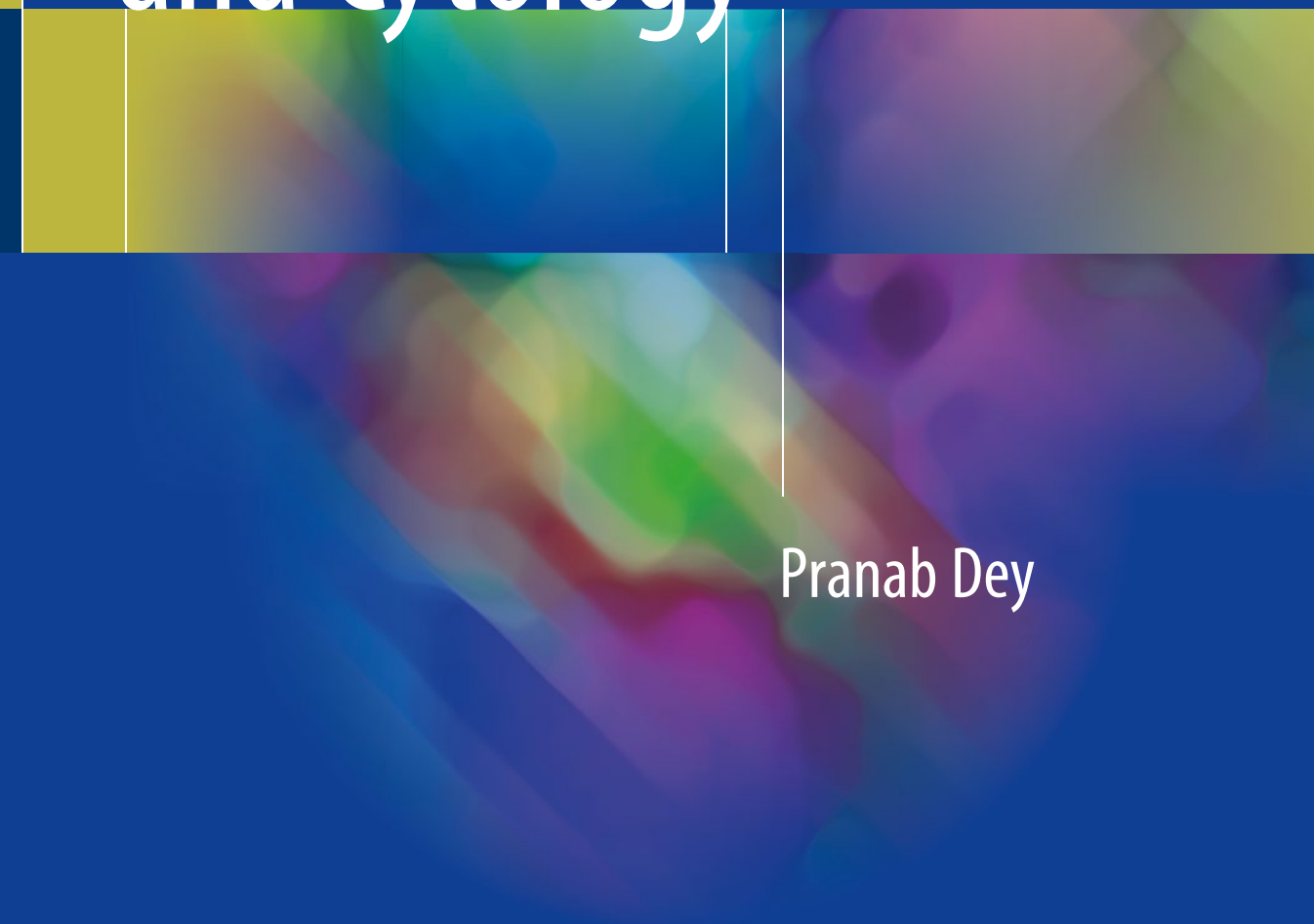
Basic and Advanced Laboratory Techniques in Histopathology and Cytology



Pranab Dey

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Dedicated to
Shree Shree Satyananda Giri,
Rini and Madhumanti

Preface

Laboratory techniques in histopathology and cytology are the foundation of the diagnostic pathology. It is extremely essential to know all the basic and advanced techniques in laboratory. This book discusses the principles, steps, and troubleshooting areas of all the essential laboratory techniques in both histology and cytology laboratories. It contains multiple illustrations, microphotographs, tables, and boxes that explain the techniques. In addition to the various advanced techniques, microscopy and quality control in the laboratory have been discussed. I hope that the book will help all the postgraduate students in pathology, practising pathologists, and laboratory technologists.

Chandigarh, India
2017 September

Pranab Dey

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My wife Rini and daughter Madhumanti constantly encouraged me during the writing of this book. They are my source of inspiration.

Lastly I wish to express my gratitude to God because without His blessing nothing can be done.

Contents

About the Author

Pranab Dey is a professor in the Department of Cytology and Gynecologic Pathology at the Postgraduate Institute of Medical Education and Research, Chandigarh. Professor Dey completed his MBBS at R. G. Kar Medical College and Hospital, Calcutta; M.D. (Pathology) at the Postgraduate Institute of Medical Education and Research, Chandigarh; and his FRCPath (Cytopathology) at the Royal College of Pathologists, London. He has conducted several research projects and has pioneered work on DNA flow cytometry, image morphometry, mono-layered cytology and cytomorphologic findings in various lesions in cytology smears. He has written numerous publications and is a member of various societies.

Abbreviations

ACEP	3Aminopropyltriethoxysilane
APAAP	Alkaline phosphatase–antialkaline phosphatase
APC	Allophycocyanin
Ab	Antibody
AR	Antigen retrieval
Acgh	Array based CGH
CEA	Carcinoembryonic antigen
CEP	Chromosome enumeration probe
CI	Colour index
CGH	Comparative genomic hybridization
CT	Computerized tomography
CFM	Confocal microscopy
CLM	Conventional light microscopy
CP	Conventional preparation
CYM	Cyan, yellow, and magenta
CK	Cytokeratin
DNA	Deoxyribonucleic acid
DSRT	Desmoplastic small round cell tumor
ddNTP	Dideoxynucleotides phosphates
DIA	Digital image analysis
EM	Electron microscope
EUS-FNAC	Endoscopic ultrasound guided FNAC
EA	Eosin Azure
EMA	Epithelial membrane antigen
ER	Estrogen receptors
EDTA	Ethylenediaminetetraacetic acid
EWS	Ewing's sarcoma
FOV	Field of view
FNAC	Fine needle aspiration cytology
FNS	Fine needle sampling
FCI	Flow cytometric immunophenotyping
FCM	Flow cytometry
FITC	Fluorescein Iso-thiocyanate
FRAP	Fluorescence recovery after photobleaching
FISH	Fluorescent in situ hybridization
FPGS	FocalPoint GS Imaging System
FFPE	Formalin fixed paraffin embedded section

GMS	Gomori methenamine silver
GLCM	Gray level co-occurrence of matrix
GFP	Green fluorescence protein
H&E	Hematoxylin and Eosin
HRP	Horseradish peroxidase
HIS	Hue saturation intensity
HCG	Human chorionic gonadotropin
ICC	Immunocytochemistry
IHC	Immunohistochemistry
Ppi	Inorganic pyrophosphate
LIS	Laboratory information service
LBC	Liquid based cytology
LSI	Locus-specific identifier probe
MRI	Magnetic resonance image
MGG	May Grunwald Giemsa
MRD	Minimal residual disease
nM	Nano micrometer
NB	Neuroblastoma
NGS	Next generation sequencing
NHL	Non-Hodgkin lymphoma
OCT	Optimum cutting temperature
OG	Orange G
PAP	Papanicolaou
PerCP	Peridinin Chlorophyll
PAS	Periodic Acid Schiff
PNET	Peripheral neuroectodermal tumor
PTAH	Phosphotungstic acid haematoxylin
PMT	Photomultiplier tube
PE	Phycoerythrin
PLAP	Placental alkaline phosphatase
PCR	Polymerase chain reaction
PR	Progesterone receptors
PSA	Prostate specific antigen
QA	Quality assurance
QC	Quality control
QI	Quality improvement
RGB	Red green blue
RCF	Relative centrifugal force
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
SEM	Scanning electron microscope
SSCP	Single strand conformation polymorphism
ssDNA	Single stranded DNA
SOP	Standard operating protocol
TIP	ThinPrep image processor
TTF-1	Thyroid transcription factor-1
TMA	Tissue microarray
TEM	Transmission electron microscope

TSA	Tyramine signal amplification
USG	Ultrasonography
VS	Virtual slides
WT	Wilms' tumor
WT 1	Wilms' tumor gene 1
Z N	Ziehl Neelsen

Part I

Basic Laboratory Techniques in Histopathology Laboratory

1 Fixation of Histology Samples: Principles, Methods and Types of Fixatives

1.1 Introduction

Fixation is the first step of any histological and cytological laboratory technique. It is the process by which the cells in the tissue are fixed in a chemical and physical state, and all the biochemical and proteolytic activities within the cells are prevented so that the cells or tissues can resist any morphological change or distortion or decomposition after subsequent treatment with various reagents. The fixation helps to maintain the tissue nearest to its original state in the living system.

1.2 Aims of Fixation

The basic aims of fixation are the following:

- To preserve the tissue nearest to its living state
- To prevent any change in shape and size of the tissue at the time of processing
- To prevent any autolysis
- To make the tissue firm to hard
- To prevent any bacterial growth in the tissue
- To make it possible to have clear stain
- To have better optical quality of the cells

1.3 Ideal Fixative

An ideal fixative should have the following qualities [1]:

1. Prevention of autolysis of the cells or tissue
2. Prevention of decomposition of the tissue by bacteria
3. Maintaining the volume and shape of the cell as far as possible
4. Consistently high-quality staining particularly routine stain such as haematoxylin and eosin stain and Papanicolaou's stain
5. Rapid action
6. Cheap
7. Non-toxic

Large number of fixatives are available in the market. Each fixative has its own advantages and disadvantages. In fact it is difficult to find a universally accepted ideal fixatives.

1.4 Tissue Changes in Fixation

The following changes may occur in tissue due to fixation (Box 1.1):

1. *Volume changes*: Fixatives may change the volume of the cells. Some fixatives such as osmium tetroxide cause cell swelling. The exact mechanism of the change in volume is not properly understood. However the volume change may be due to (a) altered membrane permeability, (b) inhibition of the enzymes responsible for respiration and (c) change of transport Na^+ ions. Formaldehyde

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Box 1.1 Change in Tissue After Fixation

- *Volume changes*
  - Shrinkage of the volume by formalin (33%).
- *Hardening of tissue*
  - Mild degree hardening may occur.
- *Interference of staining*
  - Inhibits routine stain: Osmium tetroxide inhibits haematoxylin and eosin staining.
- *Changes of optical density by fixation*
  - Nuclei may look like hyperchromatic.

may cause shrinkage of the volume by 33%. In an experiment Bahr et al. noted that the shrinkage of tissue is inversely proportional to the formaldehyde concentration [2]. Similarly glutaraldehyde also causes significant tissue shrinkage. However when glutaraldehyde and osmium tetroxide are used as fixations in epoxy resin then 70% increased of cell size is noted.

2. *Hardening of tissue*: The fixation changes the consistency of the tissue, and some amount of hardening occurs due to fixation.
3. *Interference of staining*: Fixation may cause hindrance of staining of enzymes. Formaldehyde inactivates 80% of ribonuclease enzyme [3]. It has been noted that osmium tetroxide inhibits haematoxylin and eosin staining.
4. *Changes of optical density by fixation*: The fixation may cause the change of optical density of the nuclei, and the nuclei may look like condensed and hyperchromatic [4].

1.5 Types of Fixation

The fixative can be classified on the basis of the following criteria (Table 1.1):

A. Nature of fixation
B. Chemical properties
C. Component present
D. Action on tissue protein

Table 1.1 Types of fixation and classification of fixatives

Types of fixative	Classification
A. Nature of fixation	• Immersion fixation
	• Coating fixation
	• Vapour fixation
	• Perfusion fixation
	• Freeze-drying
	• Microwave fixation
B. Chemical properties	• Aldehyde: formaldehyde, glutaraldehyde
	• Oxidising agent: osmium tetroxide
	• Protein denaturing agent: ethyl alcohol, methyl alcohol
	• Cross-linking agents: carbodiimide
	• Miscellaneous: picric acid
C. Component present	1. Simple(only one chemical present)
	• Formaldehyde
	• Ethyl alcohol
	• Glutaraldehyde
	• Picric acid
	• Osmium tetroxide
	2. Compound (more than one chemical present)
	• Bouin's fluid
	• Carnoy's solution
D. Action on protein	1. Coagulative: ethyl alcohol, picric acid
	2. Noncoagulative: formaldehyde, osmium tetroxide, glutaraldehyde

1.5.1 Description of Nature of Fixation

1. *Immersion fixation*: This is the commonest way of fixation in the laboratories. In this technique the whole specimen is immersed in the liquid fixative such as tissue samples are immersed in 10% neutral buffered formalin or cytology smear in 95% ethyl alcohol.
2. *Coating fixation*: This is commonly used in the cytology samples. The spray fixative is used for easy transportation of the slide. The main advantages of spray fixatives are:
   (a) Fixation of the cells
   (b) To impart a protective covering over the smear
   (c) No need to carry liquid fixative in bottle or jar

The spraying over the smear should be smooth and steady, and the optimum distance of 10–12 inches should be maintained between the nozzle of the spray and the smear. The spray fixative usually consists of alcohol and wax. Therefore, this wax should be removed before the staining procedure.

3. *Vapour fixation*: In this type of fixation, the vapour of chemical is used to fix either a smear or tissue section. The commonly used chemicals for vapour fixation are formaldehyde, osmium tetroxide, glutaraldehyde and ethyl alcohol. The vapour converts the soluble material to insoluble material, and these materials are retained when the smear comes in contact with liquid solution.
4. *Perfusion fixation*: This is mainly used in research purpose. In this technique the fixative solution is infused in the arterial system of the animal, and the whole animal is fixed. The organ such as the brain or spinal cord can also be fixed by perfusion fixation.
5. *Freeze-drying*: In this technique the tissue is cut into thin sections and then rapidly frozen into a very low temperature. Subsequently the ice within the tissue is removed with the help of vacuum chamber in higher temperature (−30 °C).

Steps:

- At first the thin cut tissue section is rapidly frozen at −160 °C by immersing it into liquid coolant. This is known as "quenching". The commonly used fluids in the quenching bath are liquid nitrogen, propane and isopentane. Alternatively the tissue section can be frozen by keeping it in close contact with chilled metal.
- In the next step, the ice within the tissue is removed by placing the tissue in the vacuum chamber in higher temperature (−30 to −50 °C). The water of the solid tissue is removed by sublimation. The water vapour is absorbed by a suitable drying agent.
- In the final step, the tissue is gradually warmed to 4 °C and is finally impregnated with the embedding medium.

Freeze-drying technique is useful mainly to study the soluble material and very small molecules.

Advantages:

- Excellent for enzyme study
- No change of proteins
- No shrinkage of tissue
- Preservation of glycogen

6. *Microwave fixation* (Box 1.2)

Basic principle: Microwave is a type of electromagnetic wave with frequencies between 300 MHz and 300 GHz, and wavelength varies from centimetre to nanometre. Scientific and medical microwave ovens operate with a frequency of 2.45 GHz and 0.915 GHz, respectively. The electromagnetic field is created by the microwave, and the dipolar molecules such as water rapidly oscillate in this electromagnetic field. This rapid kinetic motion of these molecules generates uniform heat. The generated heat accelerates the fixation and also other steps of tissue processing. The most important characteristic of microwave heat generation is homogeneous increase of temperature within the tissue, and every part of the tissue is heated.

Box 1.2 Microwave Fixation of Tissues

- *What it is*: Electromagnetic wave with frequencies between 300 MHz and 300 GHz.
- *Mechanism*: Microwave creates electromagnetic field, and the dipolar molecules rapidly oscillate generating heat by kinetic motion.

Advantages:

- Uniform heat production
- No volume change of tissue
- Good for electron microscopy after osmium tetroxide fixation
- Facilitates fixation and other laboratory steps
- Preservation of the tissue antigen

Disadvantages:

- Tissue in the formalin for microwave fixation may produce toxic gas and overhead hood is required.
- Heat injury may occur from microwave.

Applications:

- In routine surgical pathology laboratory
- Electron microscopy
- Urgent processing of special biopsies

Factors controlling the temperature rise: The rise of temperature in the microwave heated media depends mainly on:

- The dielectric property of the media
- Thermal properties of the material
- Radiation level
- Orientation and shape of the object

Advantages: The main advantages of microwave fixation are:

- Rapid processing.
- No change in volume of tissue.
- Preservation of the tissue antigen and good for immunohistochemistry.
- It facilitates the staining reaction without any bad effect.

Disadvantages:

- The tissue immersed in formalin during microwave fixation may generate large amount of toxic gas. Therefore overhead hood is required for the removal of this toxic substance from the microwave.
- Chances of heat injury

Applications:

- In routine surgical pathology laboratory
- Electron microscopy after osmium tetroxide fixation
- Urgent processing of biopsy (e.g. kidney biopsy)

1.6 Essential Precautions for Fixation in General

Certain essential precautions are necessary for proper fixation:

- The tissue should be free from excessive blood before putting it into fixative.
- Tissue should be thinly cut in 3–5 mm thickness.
- The amount of fixative fluid should be 20 times more than the volume of the tissue.
- The tissue with fixative should be in a tightly screw-capped bottle.

1.7 Mechanism of Fixation

The wet fixatives usually work as:

- *Dehydration and coagulation of protein:* Methanol and ethanol are commonly used coagulative fixatives. These two alcohols remove water from the tissue and causing destabilization of the hydrogen bonds and

thereby disruption of the tertiary structure of protein. However, the secondary structure of the protein is maintained. Ethanol is relatively stronger dehydrating agent than methanol. The ethanol and methanol start work from 60–80% concentration, respectively. The dehydrating fixative has two *disadvantages*:

- Shrinkage of the cells
- Removal of the soluble substances from the tissue

- *Cross-linking fixatives:*
  Formaldehyde: Formaldehyde in aqueous solution combines with water to form methylene hydrate, a methylene glycol:

$$CH_2O + H_2O = OHCH_2OH$$

In a long-standing position, this methylene glycol may further react with water molecules and form a polymer known as polyoxymethylene glycol. This again depolymerized in methylene glycol in a neutral buffer system. Formaldehyde reacts with various side chain of the protein and forms hydroxymethyl side group (Fig. 1.1). These compounds are highly reactive and subsequently cross-linking occurs by forming a methylene bridge. This preliminary reaction of hydroxymethyl side chain is the primary reaction, and the subsequent intermolecular and intramolecular cross-linking of the molecules occurs as a slow-growing process. This ultimately produces an insoluble product. The formalin can be removed from tissue by prolonged washing. However, once methylene bridge is formed in the tissue, the reaction is stable, and it is difficult to remove formalin from the tissue. Formaldehyde also reacts with the nucleic acid by reacting with the amino group of nucleotides.

Glutaraldehyde: It has two aldehyde groups that are separated by three methylene bridges (Fig. 1.2). The aldehyde group of glutaraldehyde reacts with amino group of the protein predominantly lysine. When one aldehyde group reacts with the amino group, the other free aldehyde group may help to cross-link. Glutaraldehyde rapidly and irreversibly cross-links the protein. The penetration of glutaraldehyde is slower than formaldehyde.

Osmium tetroxide: Osmium tetroxide (OsO_4) is mainly used as a fixative in electron microscopy. It is used alone or as a combination with other agent.

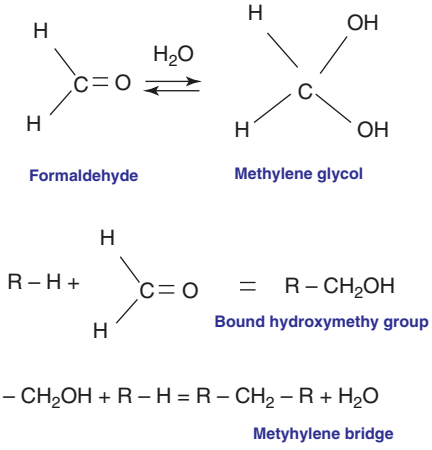

Fig. 1.1 Schematic diagram showing the mechanism of formalin fixation. Formaldehyde reacts with the side chain of the protein and forms hydroxymethyl side group. Later on these highly reactive substances form cross-linking and methylene bridges are formed. This is a stable reaction, and simple washing cannot remove formalin in this stage

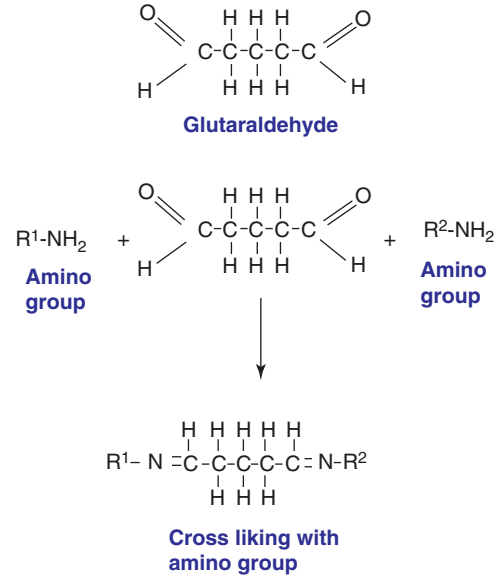

Fig. 1.2 Schematic diagram showing the mechanism of glutaraldehyde fixation. The aldehyde group of glutaraldehyde reacts with amino group of the protein. Rapid and irreversibly cross-linking of the protein takes place

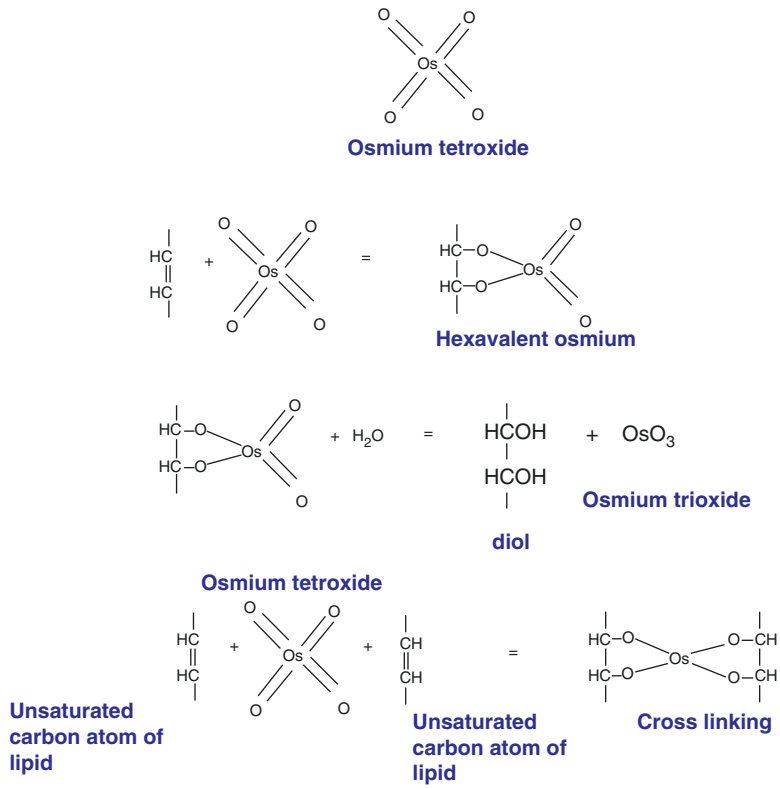

Fig. 1.3 Schematic diagram showing the mechanism of osmium tetroxide fixation. Osmium tetroxide reacts with two unsaturated carbon atom of the lipids and cross-links with them

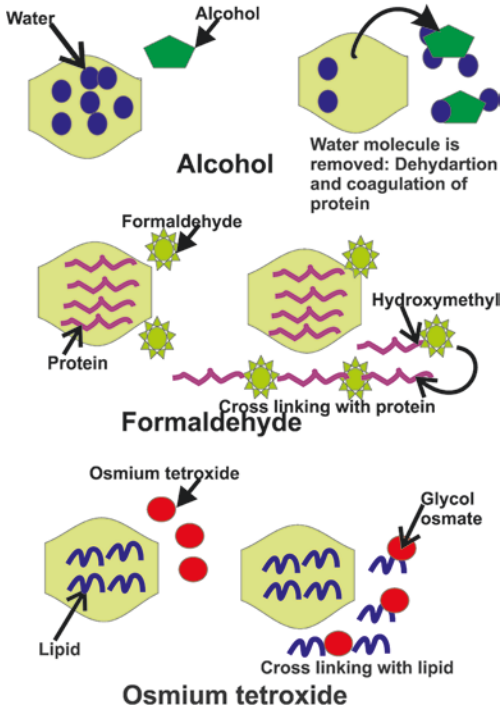

Fig. 1.4 Schematic diagram showing the mechanism of different fixatives. Alcohol works by removing water molecules from the tissue and coagulating the protein. Formaldehyde forms hydroxymethyl side group and cross-links with protein. Osmium tetroxide forms glycol osmate with lipid molecules

The compound causes oxidation of unsaturated bonds in the biological tissue particularly lipid. It converts the unsaturated fatty acid into a stable product known as glycol osmate. The tetravalent Os becomes hexavalent in this reaction. Osmic acid monoester formed in this reaction is easily hydrolysed to a diol and osmic acid (Fig. 1.3). Osmium tetroxide may react with two unsaturated carbon atom of the lipids and may cross-link (Fig. 1.3).

Figure 1.4 demonstrates the various mechanisms of fixative.

1.8 Factors Affecting Fixation

The following factors may affect the fixation (Box 1.3):

1. *Hydrogen ion concentration (pH)*: Most of the fixatives work better in neutral pH. In fact, good fixation occurs when pH remains 6–8, and no morphological distortion is seen in that range of pH. There may be changes in the ultrastructure when the pH is too low or too high. In very low pH, the NH_2 group of amino

Box 1.3 Factors Affecting Fixation

- *pH of the fixative*
  - Neutral pH is preferable.
  - pH 6–8 is the best range.
  - High acidity or alkalinity interferes fixation.
- *Temperature*
  - Room temperature suitable for routine work.
  - High temperature facilitates fixation.
  - Low temperature (0–4 °C) suitable for enzyme histochemistry.
- *Duration of fixation*
  - Depth of penetration of fixative is directly proportional to the square root of time of fixation.
  - Formalin fixes 1 mm/h.
  - Small tissue: 6 h in formalin is optimum.
  - Large tissue: 24 h is the optimum time,
  - Prolonged fixation in aldehyde: inhibition of enzymatic activity,
- *Osmolarity of the fixative solution*
  - Hypertonic: cell shrinkage
  - Hypotonic: cell swelling
  - Best: mild hypertonic (400–450 mOsm)
- *Concentration*
  - Mild lower concentration with neutral pH is preferable.
  - Very low concentration prolongs the time of fixation.
  - Higher concentration causes rapid fixation with undesirable effect.
- *Agitation*
  - Agitation increases rate of penetration.
  - Rapid agitation: damages delicate tissue.
  - Slow gentle agitation preferable.

acid is converted to NH_3, and the reaction between aldehyde groups of the fixative is reduced. Usually buffer solution is added to maintain pH of the fixative. The commonly used buffers in the fixatives are phosphate, bicarbonate, Tris and acetate. The buffers should be chosen in such a way that they should not react with the fixative.

2. *Temperature*: Room temperature is alright for tissue fixation, and there is no difference of cell morphology from 0 to 45 °C. However, the fixation time may be reduced in higher temperature (60–65 °C). At higher temperature the vibration and movement of the molecules are increased. This increases the rate of penetration of the fixative within the tissue and accelerates the process of fixation. In case of very high temperature, the antigen within the tissue may be destroyed. The enzymes are better preserved in lower temperature, and for enzyme histochemistry 0–4 °C is suitable.
3. *Duration of fixation*: The depth of penetration of fixative is directly proportional to the square root of time of fixation. The diffusibility of different fixatives may also vary:

$$D = k\sqrt{T}$$

D = depth of penetration
T = Time duration
k = Coefficient of diffusion of the fixative

The penetration rate of formalin solution is 1 mm/h. The presence of blood may hamper the penetration of the fixative. Therefore it is preferable to wash the tissue specimen thoroughly before putting it in fixative. The tissue should be

sectioned as 3–5 mm. Overall formalin fixes tissue within 24 h. Prolonged fixation may cause loss of lipid and protein and significant reduction of the enzymatic activity of the cell. This may also cause hardening of tissue.

4. *Osmolarity of the fixative solution*: Osmolality of the fixative has considerable effect on fixation. Hypertonic fixative solution causes shrinkage of the cell, whereas hypotonic fixative solution induces swelling of the cells. Electrolytes (0.9% NaCl) or sucrose may be added in the fixative to maintain the osmolarity of the fixative. Mildly hypertonic fixative (400–450 mOsm) is preferable for routine use in laboratory.
5. *Concentration*: Very low concentration of fixative prolongs the time of fixation, and higher concentration causes rapid fixation. However, higher concentration of fixative may cause tissue hardening, tissue shrinkage and artefactual changes. Mildly lower concentration of fixative with neutral pH is needed for proper fixation. Optimal concentration of formaldehyde is 10%.
6. Agitation: Agitation increases the rate of penetration and therefore rapidity of fixation. Optimum agitation is needed as slow agitation may have no effect of fixation, whereas rapid agitation may have detrimental effect on delicate tissue.

1.9 Commonly Used Fixatives in the Laboratory

1.9.1 Formaldehyde

Pure formaldehyde vapour dissolved in the water is available as formaldehyde in 37–40% concentration. This is also known as formalin and is considered as 100% formaldehyde. In laboratory 10% of this formalin is used to make neutral buffered formalin for routine laboratory fixative (Box 1.4).

Rate of penetration: Formalin penetrates approximately 1 mm/h and usually 24 h is needed for fixation of a 1 cm^3 tissue.

Volume of formalin: For proper fixation the specimen should be sliced in 5 mm apart, and the amount of formalin should be 20 times the volume of tissue. The specimen should be completely immersed in formalin and should not be in direct contact with the container. There should be a formalin soaked clothes in between the container and the tissue.

Removal of formalin from the tissue: As the cross-linking of the amino acids and proteins is a slow process, so if the tissue is washed for 24 h in water, then 50% of formalin from the tissue is removed.

Precaution: Formaldehyde is irritant to the eye and skin and toxic for inhalation. It is a carcinogenic element.

Advantages:

- The penetration rate of formalin is high.
- Cell morphology well preserved in formalin.
- Cheap.
- Stable.
- Easy to make the solution.
- Formalin is effective fixation for routine laboratory staining of the tissue.

Disadvantages;

1. Slow fixation.
2. Formalin reaction with the tissue is reversible, and it can be removed by washing.
3. Formalin fails to preserve acid mucopolysaccharides.
4. Highly vascular tissue may have dark-brown granules (artefact)
5. Exposure to the skin may cause dermatitis.
6. Chronic inhalation may cause bronchitis.

1.9.2 Preparation of Different Formalin Solution

A. *10% neutral buffered formalin*:
   - Formaldehyde, 40%: 100.0 ml
   - Distilled water: 900.0 ml
   - Sodium dihydrogen phosphate: 4.0 g
   - Disodium hydrogen phosphate: 6.5 g

Box 1.4 Formaldehyde Fixative

- Commercially available as 40% concentration (considered as 100% formalin)
- Ten percent of this formalin is used to make neutral buffered formalin.

Mechanism: It reacts with various side chain of the protein and forms hydroxymethyl side group that subsequently cross-link to form a methylene bridge. Subsequent intermolecular and intramolecular cross-linking of the molecules occurs and ultimately produces an insoluble product.

Rate of penetration: Formalin penetrates approximately 1 mm/h.

Volume of formalin: The amount of formalin should be 20 times the volume of tissue.

Advantages:

- High penetration rate
- Well-preserved cell morphology
- Cheap
- Stable

Disadvantages:

1. Slow fixation.
2. Formalin reaction is reversible, and it can be removed by washing.
3. Fails to preserve acid mucopolysaccharides.
4. Not good for staining of fat and enzymes.
5. Highly vascular tissue may have dark-brown granules.
6. Exposure to the skin may cause dermatitis.
7. Chronic inhalation may cause bronchitis.

B. *Preparation of 10% formal saline*:
   - Formaldehyde, 40%: 100.0 ml
   - Sodium chloride: 9 g
   - Distilled water: 900.0 ml

C. *Formal ethanol fixative:*
   - Ninety-five percent ethyl alcohol: 20 ml
   - Formaldehyde, 40%: 10 ml

1.9.3 Glutaraldehyde

Glutaraldehyde is used as a fixative for electron microscopy because it fixes and preserves the ultrastructure. The fixation occurs due to the extensive cross-linking of the proteins. The penetration power of glutaraldehyde is poor and therefore only a small piece of tissue should be used for fixation. Glutaraldehyde does not react with lipid or carbohydrate, and therefore it should be used in combination with the other fixative.

Advantages:

1. Better fixation of ultrastructure.
2. Less cell shrinkage.
3. Preservation of protein is better.
4. Good cross-linking with collagen.
5. Less irritating.

Disadvantages;

1. Poor penetration and tissue should be less than 0.5 mm thick.
2. Less stable compound.
3. No lipid fixation.
4. Glutaraldehyde polymerizes above pH 7.5.
5. Costly.

For the purpose of electron microscopy:

Glutaraldehyde is used 2.5% glutaraldehyde in 100 mM phosphate buffer at pH 7.0.

Glutaraldehyde comes commercially as 25% or 50% solutions in 10 ml.

1.9.4 Osmium Tetroxide

Osmium tetroxide is used for fixation in electron microscopy. It reacts with unsaturated bonds in the lipid molecules and fixes them. The penetration of the osmium tetroxide in the tissue is poor, and if it is used alone, then a good amount of protein and carbohydrate may be lost during fixation.

Advantages:

1. This is a very good fixative for lipid.
2. It preserves cytoplasmic organelles such as Golgi bodies and mitochondria,
3. Does not make the tissue hard,

Disadvantages:

1. It does not fix the proteins and carbohydrates and therefore it should be used in combination with other fixative.
2. Osmium tetroxide may react with ribose group and may cause clumping of DNA. This can be prevented by pretreatment with potassium permanganate or post fixation with uranyl acetate.
3. Poor penetration in the tissue.
4. Tissue swelling may occur.
5. Toxic and volatizes at room temperature producing harmful vapour. This vapour is toxic to the eye and respiratory tract.
6. Expensive.

Laboratory use: It is commercially available in sealed vial 0.1–1 g. Aqueous solution of 4% OsO_4 is made. This should be stored in clean glass vial away from sunlight. In laboratory 2–4% OsO_4 in buffer solution of pH 7.2 is used.

1.9.5 Methyl and Ethyl Alcohol

Methyl alcohol (methanol) and ethyl alcohol (ethanol) are used as dehydrating agent, and these two alcohols are used mainly as fixatives of cytology smears. The tissue or smear containing water should not be put directly in the higher concentration of alcohol as it may cause distortion of the cells due to rapid rush of fluid from the cell. Therefore graded alcohol should be used for dehydration.

Laboratory use: 95% ethyl alcohol for fixation

Reagent preparation:

Absolute alcohol: 950 ml
Water: 50 ml
Time of fixation: 15–30 min

1.9.6 Acetone

It is mainly used for enzyme study and immunocytochemistry. It is a poor fixative for morphological preparation as it causes significant cell shrinkage. Acetone works by dehydration of cells. Cold acetone is used at 4 °C for fixation.

1.9.7 Bouin's Fixative

Bouin's solution contains picric acid. This is an excellent fixative for glycogen. It reacts with protein and forms protein picrate. The tissue penetration rate of picric acid is high, and it fixes small tissue biopsy within 3–4 h. Bouin's fixative is not suitable for DNA quantitative study as it damages the cell membrane and causes hydrolysis of nuclei acid.

Advantages:

1. It is a good fixative for connective tissue and glycogen.
2. Rapid penetration rate.

Disadvantages:

1. It produces yellow stain to the tissue.

Removal of yellow colour:

1. The tissue should be washed thoroughly in 70% ethanol.
2. This yellow colour can be removed by dipping the tissue in lithium carbonate in 70% alcohol.

Bouin's solution preparation:

Picric acid solution (1% in distilled water): 15 ml
Formaldehyde stock solution (40%): 5 ml
Glacial acetic acid: 1 ml

1.10 Mercury Salt-Containing Fixatives

Among the heavy metals, mercury is commonly used as fixative. This is a rapidly acting fixative. Mercury is a poisonous substance and should be used carefully. Mercury containing fixatives may corrode the metal so the fixative should be kept in glass container.

1.10.1 Zenker's Fluid

It is a good fixative for nuclear chromatin and collagen.
Preparation
Mercuric chloride: 50 g
Glacial acetic acid: 50 g
Potassium dichromate: 25 g
Distilled water: 950 ml

1.10.2 Helly's Fluid

This is a good cytoplasmic fixative. It takes nearly about 12 h for 3 mm tissue to fix.

Preparation
Solution A

Distilled water: 250
Potassium dichromate: 6.3 g
Mercuric chloride: 12.5 g
Sodium sulphate: 2.5 g
Solution B
Thirty-seven percent formaldehyde solution
Just before use mix 95 ml of Solution A with 5 ml of Solution B.

1.10.3 B5 Fixatives

Stock A solution
Mercuric chloride: 12 g
Sodium acetate: 2.5 g
Distilled water: 200 ml
Stock B solution
Thirty-seven percent formaldehyde solution
Before use mix 20 ml stock solution A with 2 ml stock B.

Table 1.2 highlights the comparison of different fixatives.

1.10.4 Fixatives of Choice

The choice of fixatives is very important for specific purposes. In case of routine histopathology tissue, the best fixative is 10% neutral buffered formalin. Similarly for electron microscopy and immunocytochemistry, the fixative of choice is glutaraldehyde solution and 10% neutral buffered formalin, respectively. Table 1.3 highlights the fixative of choice for different technique.

The different types of fixative are used according to the tissue and have been highlighted in Table 1.4.

Fixative of choice may be different according to chemical compounds to demonstrate (Table 1.5).

Table 1.2 Comparison of different fixatives

Fixative	Ingredients	Advantages	Disadvantages	Applications
Buffered formalin (10%)	– Formaldehyde – Water – Sodium dihydrogen phosphate – Disodium hydrogen phosphate	– High penetration rate – Cell morphology well preserved – Cheap – Stable	– Slow fixation – Fails to preserve acid mucopolysaccharides – Dark-brown granules in vascular tissue	– Effective for routine laboratory staining
Glutaraldehyde	– Glutaraldehyde – Phosphate buffer	– Better fixation of ultrastructure – Less cell shrinkage – Protein preservation better – Less irritating	– Poor penetration in tissue – Less stable – No lipid fixation – Costly	– Best for electron microscopy
Osmium tetroxide	–2–4% Osmium tetroxide in buffer solution	– Good fixative for lipid – Good for Golgi bodies and mitochondria	– Does not fix the proteins and carbohydrates – Cause clumping of DNA – Toxic and volatizes at room temperature – Costly	– Good for electron microscopy
Ethyl alcohol	– Absolute alcohol – Water	– Fast penetration	– Inflammable – Needs licence	– Good for cytology smear
Bouin's fixative	– Picric acid – Formaldehyde – Glacial acetic acid	– Rapid penetration rate – Very good for trichrome stain	– Produces yellow stain to the tissue	– Good fixative for connective tissue and glycogen
Zenker's fluid	– Mercuric chloride – Glacial acetic acid – Potassium dichromate – Distilled water	– Rapidly acting – Even penetration	– Pigments of dichromate and mercury – Mercury is poisonous – RBC is poorly preserved	– Organ with very high vascularity such as the spleen

Table 1.3 Choice of fixative based on technique

Technique	Fixative of choice
Routine histopathology	10% neutral buffered formalin
Electron microscopy	Glutaraldehyde solution or osmium tetroxide
Immunohistochemistry	10% neutral buffered formalin, alcoholic formalin
Immunofluorescence	Unfixed cryostat
Enzyme histochemistry	Fresh frozen section

Table 1.4 Fixative of choice according to tissue

Tissue	Fixative	Time
Day-to-day sample (routine)	10% buffered formalin	Small tissue: 6 h Large tissue: 12–24 h
Lymph node	B5 solution	18 h
Gastrointestinal tract	10% buffered formalin	6 h
Testis	10% buffered formalin Or Bouin's fluid	6 h
Bone marrow	Bouin's fluid	3 h
Spleen	Zenker's fluid	6 h
Eye	10% buffered formalin	48 h

Table 1.5 Fixative of choice for different substances

Target substance	Fixative of choice
Protein	10% buffered formalin
Lipid	Frozen section or osmium tetroxide
Glycogen	Alcohol-based fixative
Mucopolysaccharide	Frozen section
Enzyme	Frozen section
DNA and RNA	Alcohol-based fixative
Iron	Alcohol-based fixative

1.11 Fixation Artefact

The following fixation artefact may be encountered in routine laboratory fixation (Box 1.5):

A. Formalin Pigment

Unbuffered formalin is kept for long time and is converted into formic acid that reacts with haemoglobin derivatives of the blood and produces acid formaldehyde haematein which is an insoluble brownish-black granular retractile birefringent pigment (Fig. 1.5) (Box 1.6).

Removing the pigment:
Steps:

1. The section is immersed in xylene followed by alcohol to bring in water.
2. Subsequently the section is immersed in 1.8% picric acid in absolute ethyl alcohol for 15 min.
3. It is then washed thoroughly.
4. Section is re-stained.

Box 1.5 Fixation Artefact

- *Formalin pigment*: Insoluble brownish-black granular retractile birefringent pigment due to reaction of formalin with haemoglobin derivatives.
- *Mercury pigments*: Dark-brown irregular deposit.
- *Fuzzy staining*: Due to improper fixation.
- *Prolonged fixation*: Shrinkage of the tissue causes tissue separation and empty spaces.
- *Dichromate deposit*: This deposit may occur after dichromate fixation if the tissue is not washed properly.

Box 1.6 Formalin Pigment

- *Colour*: Brownish black.
- *Nature*: Granular birefringent refractile.
- *Position*: Extracellular.
- *Mechanism of formation*: Formic acid reacts with haemoglobin derivatives of the blood and produces acid formaldehyde haematein.
- *How to avoid*: Use buffered formalin.
- *How to remove*: Treat with 1.8% picric acid in absolute ethyl alcohol for 15 min.

B. *Mercury Pigments*: When tissue is fixed by mercuric chloride, it produces a dark-brown irregular deposit.

Location: Throughout the tissue

Removal: Application of iodine converts it into mercuric iodide which is removed by sodium thiosulphate.

C. *Fuzzy Staining*

Appearance: Here the nuclear and cytoplasmic details are obscured and the section looks fuzzy or hazy.

Cause: Improper fixation either due to insufficient fixative or too little time in fixative.

D. *Prolonged fixation*: Prolonged fixation cause shrinkage of the tissue followed by separation. The tissue may show holes or empty spaces within it (Fig. 1.6).

E. *Dichromate deposit*: If the tissue is not properly washed after dichromate fixation, then the chromium salt may form. This chrome salt reacts with alcohol, and insoluble yellow-brown precipitate may appear.

Removal: By 1% hydrochloric acid in 70% alcohol for 30 min

Troubleshooting in fixation is highlighted in Table 1.6.

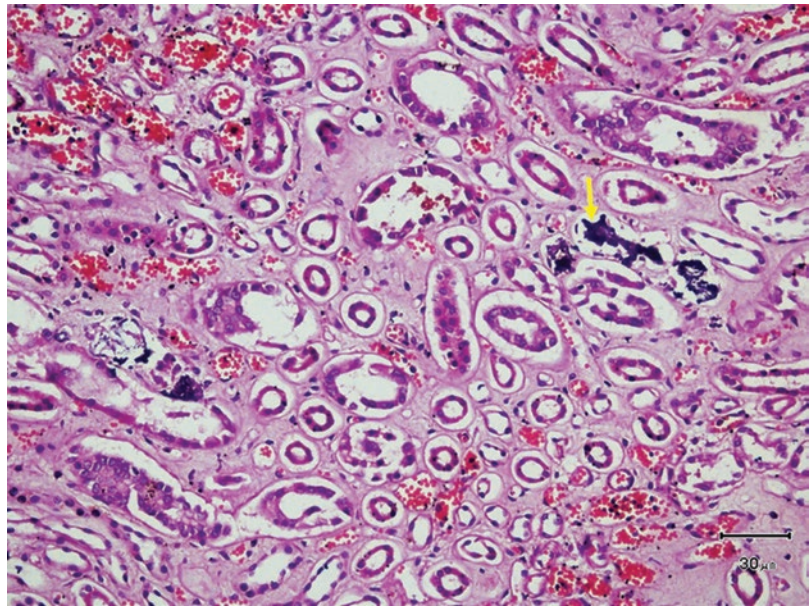

Fig. 1.5 Microphotograph shows brownish-black granular formalin pigment. This is refractile birefringent pigment (haematoxylin and eosin stain ×400)

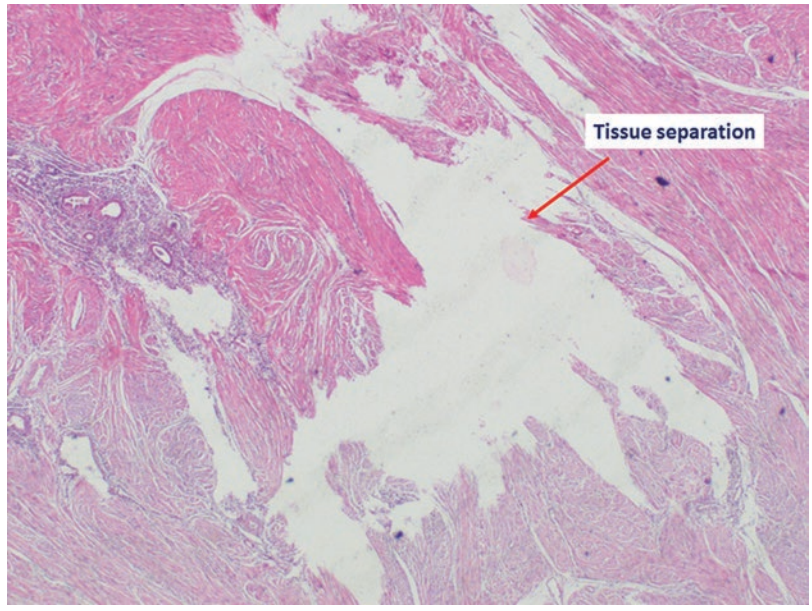

Fig. 1.6 Tissue separation due to prolonged fixation. The tissue shows holes and empty spaces within it (haematoxylin and eosin stain ×200)

Table 1.6 Troubleshooting in fixation

Problems	Cause	Remedies
Nuclear margin is indistinct and nuclei are fuzzy with bubbling	Incomplete fixation	– Check the concentration of formalin – Keep more time in formalin for fixation – Cut thin section for fixation – Do not put too many cassettes together
Tissue shrinkage with large artefactual spaces	– Poor fixation – Prolonged fixation	– Proper fixation time – Check fixative concentration – Immediately fix the tissue after biopsy
Insoluble brownish-black granular pigment	Formalin pigment due to acid formaldehyde haematein formation by reaction with blood	Use buffered formalin
Intraepithelial cleft formation	Formalin may evaporate, and calcium carbonate is precipitated	Keep formalin in closely capped bottle

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Processing of Tissue in Histopathology Laboratory

2

The next step after fixation is the processing of tissue. This is also a very important step because poor processing of tissue may significantly affect the section cutting and staining.

Aims of tissue processing: The basic aim of tissue processing is to provide sufficient rigidity to the tissue so that it can be cut into thin section for microscopic examination.

Principle of processing: In tissue processing the water within the tissue is removed, and another medium (usually paraffin wax) is impregnated in the tissue that provides the adequate support to the tissue. Therefore the essential steps in tissue processing (Fig. 2.1):

1. *Dehydration*: In this step water is removed from the tissue. Water is immiscible with wax, and therefore to infiltrate the tissue with wax, it is necessary to remove water.
2. *Clearing*: This is needed to clear the dehydrating agent and to facilitate the transition of dehydration and impregnation stage. The clearing substance is usually miscible to both dehydrating agent and impregnating medium.
3. *Infiltration and impregnation*: In this stage, the tissue is infiltrated with a supporting medium which is suitable to provide adequate rigidity of the tissue to make thin section.

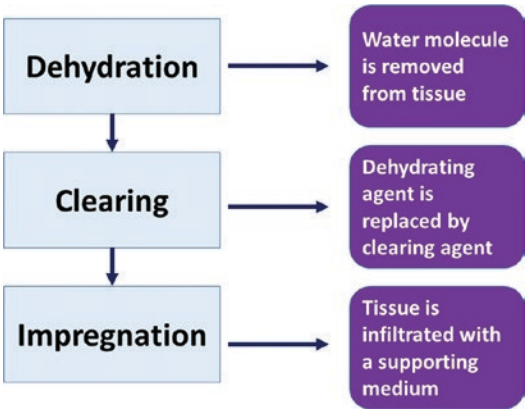

Fig. 2.1 Schematic diagram showing overview of processing. The basic three steps are dehydrating, clearing, and embedding. Removal of water from the tissue is known as dehydration. This is followed by clearing of the dehydrating agents by the process clearing. In the final stage tissue is impregnated with an embedding medium

2.1 Factors that Influence Tissue Processing

The following factors influence the tissue processing (Box 2.1):

1. *Size of the tissue sample*: The optimum size of the tissue is very important for effective

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Box 2.1 Influencing Factors of Tissue Processing

- *Size of the tissue*:
  - The smaller the size, the better the processing.
- *Agitation*:
  - Agitation facilitates the contact of tissue with fresh solution.
- *Heat*:
  - Increases the better penetration of fluid.
- *Viscosity*:
  - The higher the viscosity of the medium, lower the penetration.
- *Negative pressure*:
  - Negative pressure removes trapped air in the tissue.
  - Removal of clearing agent by increasing volatility.

processing. The smaller the size of the tissue, better is the infiltration of the embedding medium. Optimum thickness of the tissue should be kept as 3–4 mm only.

2. *Agitation*: The tissue gets better contact with the surrounding medium if it is completely immersed and gently agitated. The agitation causes continuous removal of the fluid from the surface by fresh medium. This has better effect of action of fluid on the tissue. Most of the commercial tissue processors have the facility of agitation. It is important to note that too rapid agitation may damage the soft and delicate tissue.
3. *Heat*: Heat increases the rate of penetration of the fluid within the tissue, whereas low temperature impedes the whole process. The present commercial tissue processors have the facility to heat the tissue in all stages of processing. Overheating may produce hard and brittle tissue.
4. *Viscosity*: The viscosity of the embedding media also affects the processing. Higher viscosity of the medium lowers the penetration rate in the tissue. Heat reduces the viscosity of the medium and helps in better penetration.
5. *Vacuum*: Application of negative pressure facilitates tissue processing [1]. Vacuum helps to remove the entrapped air from the tissue and thereby enhances the penetration of fluid within the tissue. Negative pressure also increases the volatility of the clearing agent and therefore helps to remove the fluid from the tissue.

2.2 Dehydration

Every tissue contains some amount of free or unbound water molecule. As the commonly used supporting medium (paraffin) is not miscible with water, so it is necessary to remove the free water molecule from the tissue for the successful impregnation of the supporting medium (Box 2.2). The sharp difference of the concentration gradient between the tissues and the dehydrating fluid may cause sudden rush of fluid, and this may damage the delicate tissue. Therefore the dehydration should be done gradually from low

Box 2.2 Dehydration

- Removes free or unbound water molecule of the tissue as the supporting medium (paraffin) is not miscible with water.
- Sharp difference of concentration gradient of the dehydrating fluid may damage the delicate tissue.
- Gradual dehydration is necessary.
- Too much time in the dehydrating fluid: the tissue becomes hard and brittle.
- *Routine laboratory*: 70, 90 and 100% alcohol for 2 h each.
- *Common dehydrating agents*:
  - Ethyl alcohol
  - Methylated spirit
  - Methanol
  - Butyl alcohol
  - Isopropyl alcohol
  - Dehydrating agents other than alcohol: dioxane, ethylene glycol and acetone

to high concentration of dehydration fluid. The tissue should be kept in the dehydration fluid for optimal time because too much time in the dehydrating fluid may cause the tissue hard and brittle. Too little time in dehydration fluid may be insufficient for removal of free water molecule. Thin 2–3 mm tissue needs less time in dehydration fluid than thick 5 mm tissue.

2.3 Individual Dehydrating Agent

2.3.1 Alcohol

Ethanol Ethanol or ethyl alcohol is the most popular and most commonly used dehydrating agent. This is a clear and colourless fluid. Ethyl alcohol is flammable liquid. This is a relatively rapid and efficient dehydrating agent. However, it needs licence from the government to purchase ethyl alcohol for laboratory use.

As a dehydrating agent ethyl alcohol is used in 50, 70, 90 and 100% concentration. For delicate tissue, the dehydration may be started from 30% concentration of ethyl alcohol. In routine laboratory, 70, 90 and 100% alcohol for 2 h each is sufficient for dehydration of the tissue. If tissue is immersed in the ethyl alcohol for long time, then the removal of attached water from the carbohydrate and protein molecules causes hard and brittle tissue.

Anhydrous Cupric Sulphate in Final Container Anhydrous cupric sulphate ($CuSO_4$) is a white powder that draws water from the alcohol and thereby helps in dehydration. About 1 cm layer of this powder is kept in the bottom of the container. The cupric sulphate powder should be covered with two to three layers of filter paper to prevent any colouring of the tissue. When the $CuSO_4$ becomes hydrated, the colour of the powder changes to blue. This gives warning signal to change the alcohol and the $CuSO_4$ powder.

Advantages:

- Increases the life span of alcohol
- Better dehydration
- Good indicator to change alcohol

Methylated Spirit It is also known as denatured alcohol. Methylated spirit contains 99% ethanol and 1% methanol or isopropyl alcohol.

Methanol Methanol is a clear, colourless, volatile and inflammable liquid. It can be used as a substitute of ethanol, but it is rarely used in laboratory because of its volatility and high cost.

Butyl Alcohol *n*-Butanol, isobutanol and tertiary butanol are used as dehydrating agents both in animal tissue and plant histology processing. Butyl alcohol is a slowly acting dehydrating agent and takes longer time than ethyl alcohol for dehydration. However the tissue shrinkage is less by butyl alcohol.

Isopropyl Alcohol Isopropyl alcohol is available as isopropanol (99.8%). This is miscible with water and liquid paraffin. It is a relatively rapid acting, non-toxic dehydrating agent causing minimal tissue shrinkage. It is a good lipid dissolving solvent.

2.3.2 Dehydrating Agents Other than Alcohol

Dioxane This is 1,4-diethylene dioxide. Dioxane is miscible with both water and molten paraffin wax. This is a rapidly acting dehydrating agent and produces very little shrinkage. Tissue can be kept in dioxane without any harm. Dioxane liberates highly toxic gas and proper ventilation is mandatory for its use.

Ethylene Glycol It is also known as ethylene glycol monoethyl ether or cellosolve. It is a colourless and odourless fluid. Cellosolve is a rapidly acting dehydrating and tissue can be kept in it for long duration.

Acetone Acetone is a colourless and inflammable liquid with a pungent ketonic smell. It is

Table 2.1 Comparison of different dehydrating agents

Dehydrating agents	Advantages	Disadvantages
Ethyl alcohol	• Rapid and efficient dehydrating agent	• Needs licence from the government • Inflammable • Hard and brittle tissue if kept for long time
Methanol	• Equally effective as ethanol	• Volatile • High cost
Isopropyl alcohol	• Relatively rapid action • Non-toxic • Minimal tissue shrinkage	• Not possible to use in celloidin technique
Dioxane	• Rapid action • No shrinkage of tissue	• Highly toxic gas is generated
Ethylene glycol	• Rapid • No graded solution is needed • Tissue can be kept in it for long time	• Very expensive • Clearing agent is needed
Acetone	• Rapid action • Cheaper than ethanol • Good for fatty tissue processing	• Quickly evaporates • Inflammable • Prolonged use may cause shrinkage and brittleness of tissue

miscible with both water and alcohol. Acetone produces tissue shrinkage and prolonged use may cause brittleness of tissue. It is best used in fatty tissue processing.

The various dehydrating agents are compared in the Table 2.1.

2.4 Clearing

After the removal of free water molecule from the tissue, the next step of processing is to remove the dehydrating agent itself from the tissue because many dehydrating agents are not miscible with the impregnating material (paraffin wax). The clearing agent should be miscible with both the dehydrating agent and the embedding medium. The refractive index of the clearing agent is similar to the tissue, and therefore it gives clear appearance of the anhydrous tissue (Box 2.3). So the completely transparent tissue indicates the terminal point of the clearing process. Any opacity of tissue signifies incomplete dehydration.

Selection of appropriate clearing agent: This depends on:

- *Type of tissue*: large tissue takes more time than smaller tissue
- *Type of processor*: manual versus automatic
- *Processing condition*: temperature and vacuum
- *Speed of removal* of dehydrating agent
- *Ease of replacement by molten wax*
- *Safety factors*: flammability and toxicity
- *Cost*

The clearing agent with low melting point is easily and quickly removed by the molten wax. Whereas, clearing agent with high melting point takes time to be removed by embedding medium. Clearing agent with high viscosity has low penetration rate. Prolonged exposure of the tissue in clearing agent may make the tissue brittle and more friable. Therefore optimal time for clearing is necessary. The amount of clearing agent should be 40 times of the volume of tissue for clearing.

2.4.1 Individual Clearing Agent

Xylene This is the most commonly used clearing agent in the laboratory. This is a clear and inflammable liquid. The small pieces of tissue are cleared rapidly by xylene within 30–60 min. Prolonged exposure to xylene may make the tissue hard and brittle.

Box 2.3 Clearing

- *Aims of clearing*:
  - Removal of dehydrating agent (e.g. alcohol) to facilitate impregnation of paraffin wax
  - To make the tissue clear and improve the microscopic examination
- *Ideal clearing agent*:
  - Low viscosity and high penetration rate
  - Low melting point
  - Miscible with both alcohol and molten wax
  - No tissue damage
  - Less toxic
  - Less inflammable
  - Cheap
- *Selection of appropriate clearing agent*: Type of tissue, type of processor, processing condition (such as heat, vacuum) safety factors and cost
- *Volume of clearing agent*: 40 times the volume of the specimen
- *Total duration*:
  - *Smaller biopsy*: 1 h
  - *Larger tissue*: Three changes in xylene or toluene 60 min each
- *End point detection*: Tissue becomes transparent
- *Prolonged exposure to clearing agent*: The brittle and more friable tissue
- *Different clearing agents*: Xylene, toluene, chloroform, amyl nitrate, cedarwood oil and limonene

Table 2.2 Comparison of clearing agents

Properties	Xylene	Toluene	Chloroform	Esters
Tissue shrinkage	Yes	Yes	Minimum	No
Tissue hardening	Yes	No	No	No
Inflammable	Yes	Yes	No	Yes
Harmful effect	Irritant but less harmful	Irritant	Dangerous toxic gas liberates in heating	Safe
Cost	Cheap	Cheap	Very expensive	High cost

Toluene It has almost similar properties as that of xylene. However it does not make the tissue hard even after prolonged exposure, and its action is slightly slower than xylene. Toluene is also flammable and toxic.

Chloroform It is highly volatile, non-inflammable, expensive and toxic agent. The penetrating power of chloroform is slower than xylene. However, it does not cause any tissue shrinkage and is mainly used in the uterus, muscle and other dense tissue. Presently chloroform is rarely used in laboratory.

Table 2.2 compares the different clearing agents commonly used in laboratories.

2.4.2 Other Clear Agents

Esters The different esters are amyl nitrate, methyl salicylate and methyl benzoate. These are less toxic and may be used in manual processing. They do not cause tissue hardening even under prolonged exposure.

Cedarwood Oil This is an expensive rapid clearing agent and mainly used in clearing dense tissue.

Limonene This is a clear liquid. It does not cause any tissue hardening. However, the removal of limonene from the tissue by paraffin wax is difficult.

2.5 Infiltration and Embedding

This is the next step after clearing. The clearing agent within the tissue is removed by the process of diffusion. The tissue space is now infiltrated with the embedding media. Usually molten wax is used as the embedding medium. After cooling in room temperature, the molten wax is solidified to provide support for cutting into thin section (Box 2.4).

Ideal impregnating medium: An ideal impregnating medium should have following qualities:

- Miscible with clearing agent
- Liquid in higher temperature (30–60 °C) and solid in room temperature
- Homogenous and stable
- Non-toxic and cheap
- Transparent
- Fit for sectioning the tissue

The time duration and the number of changes required for the impregnation in tissue depends on:

1. *Size of tissue*: Thicker large tissue takes more time to impregnate with the embedding medium. It also contains more clearing agent to remove.
2. *Type of tissue*: Hard tissue such as bone and cartilage takes more time for embedding than soft tissue.
3. *The type of clearing agent*: Certain clearing agents are easy to remove than others. Such as xylene and toluene are easy to remove than cedarwood oil.
4. *Type of processing*: Vacuum embedding enhances impregnation.

2.5.1 Different Impregnating Medium

2.5.1.1 Paraffin Wax

Paraffin wax is a type of hydrocarbon and is produced as by-product during refining of crude petroleum. This is the most popular universally accepted embedding medium for tissue processing. This is non-toxic and inexpensive medium. The melting point of paraf-

Box 2.4 Impregnation of Embedding Medium

Aims: To provide support to the tissue.

Principle: Clearing agent is removed by the process of diffusion, and the tissue space is now infiltrated with the embedding media.

Ideal impregnating medium:

- Miscible with clearing agent
- Liquid in higher temperature and solid in room temperature
- Homogenous and stable
- Non-toxic and cheap
- Transparent
- Fit for sectioning the tissue

Time duration and the number of changes of embedding medium:

- Size of tissue: Large versus small.
- Type of tissue: Hard versus soft.
- The type of clearing agent: Cedarwood oil takes longer time.
- Type of processing: Vacuum processing accelerates.

Different embedding medium: Paraffin wax and dimethyl sulphoxide.

fin wax varies from 39 °C to 70 °C. The wax is sold according to its melting point. Paraffin wax with low melting point is soft in room temperature, whereas paraffin wax of higher melting point is much harder in consistency. Therefore, it is necessary to have paraffin wax that has suitable melting point to get good ribbon of tissue. In this Indian subcontinent, the paraffin wax with melting point around 60 °C is the most suitable for laboratory use. Total 3–4 h' time in paraffin wax is sufficient for impregnation of tissue by wax.

Advantages of paraffin wax:

- Tissue block can be stored for long duration.
- Non-toxic.
- Cheap.
- Safe.

Disadvantages of paraffin wax:

- It may cause tissue shrinkage and hardening in case of prolonged impregnation.
- Paraffin wax takes long duration for the impregnation of the bone and eye.

2.5.1.2 Additives and Modification of Paraffin Wax

To alter the physical characteristics of paraffin wax, the following modifications may be done:

1. *To increase hardness*: addition of stearic acid
2. *Reduction of melting point*: addition of phenanthrene
3. *Improving adhesiveness with tissue and wax*: addition of 0.5% of ceresin

Dimethyl Sulphoxide (DMSO) The addition of small amount of DMSO in paraffin wax reduces the infiltration time of the wax and removes the residual clearing agent. It produces a homogenous matrix and better support.

2.6 Tissue Processing Methods

Tissue processing can be done by simple manually or by automated processor. Manual tissue processing is done only in a small laboratory with a handful number of tissue. Automated tissue processor is widely used in laboratories.

Automated tissue processor: The basic principle of tissue processor is to transfer the tissue in different fluid for a specified time in a desired environment. There are two types of tissue processor:

1. Tissue transfer processor
2. Fluid transfer processor
    1. *Tissue transfer processor* (Fig. 2.2): In this system the bucket of tissue is transferred from one carousel to other after a specified time. There are several containers with reagents. Tissue remains in a basket with 30–100 cassettes. The basket containing the tissue is submerged in the specific container for a particular time and then transferred to the next container automatically. A gentle agitation is created by vertical oscillation or by rotatory movement of the tissue basket. The time schedule and transfer of tissue in each container are determined by a microprocessor.
    2. *Fluid transfer processor* (Fig. 2.3): This is a completely closed processor. Here the tissue is kept in the container, and the container is periodically filled with particular fluid. After a certain period, the fluid is pumped out from the container containing the tissue. It is again filled with the fluid required for the next step. In this processor each step can be customized for vacuum, temperature, and time duration.

Fig. 2.2 Tissue transfer automatic tissue processor. Here the whole bucket containing tissue is automatically transferred to the next fluid station

Fig. 2.3 Fluid transfer advanced automatic tissue processor. Here the fluid itself is changed automatically, and the bucket remains static

Advantages:

1. Vacuum pressure makes the system faster and efficient.
2. In this closed system, there is no chance for tissue drying.

2.7 Overall Precautions of Tissue Processing

1. The bulk of the tissue should be optimum for adequate penetration of fluid.
2. The amount of fluid should be adequate and the fluid level should be always higher than the tissue level.
3. The tissue basket and cassettes should be clean and any spillage of wax should be cleaned.
4. The temperature of the infiltrating medium should be optimum, and it is preferable to keep the temperature 3–4 °C above the melting point.
5. There should be a proper record of the change of fluid, number of tissues processed, etc.

2.7.1 Time Schedule for Overnight Processing (Fig. 2.4)

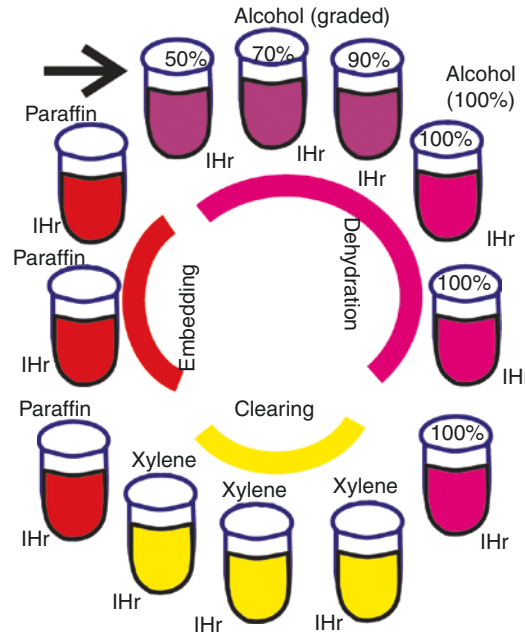

Fig. 2.4 Schematic diagram showing the time schedule of overnight processing

50% ethanol: 1 h
70% ethanol: 1 h
90% ethanol: 1 h
Absolute alcohol: 1 h
Absolute alcohol: 1 h
Absolute alcohol: 1 h
Xylene/toluene: 1 h
Xylene/toluene: 1 h
Xylene/toluene: 1 h
Paraffin wax: 1 h
Paraffin wax: 1 h
Paraffin wax: 1 h

2.7.2 Manual Tissue Processor

It is rarely used in routine laboratory. The advantage of manual processing are:

1. Small number of samples can be processed in a small laboratory.
2. Careful monitoring in each step is possible.
3. In case of emergency when the automated tissue processor is not working, one can take the help of the manual processing.
4. In case of manual processing, it is possible to select the reagents of choice with flexibility in time duration.

Table 2.3 Troubleshooting in processing

Problem	Cause	Remedies
Tissue is soft during embedding	1. Tissue is too thick during grossing 2. Reagents are saturated with water 3. Paraffin is saturated with clearing agent e.g. Xylene	1. Tissue should be properly cut 2. Change the reagents 3. Change paraffin
Tissue is coming out from the block during sectioning	Dehydration process is inadequate, so there is defective paraffin infiltration in the tissue	Change the processing program and give adequate time for dehydration
Microchattering effect around the edges of the tissue section	Excessive dehydration	Change the dehydration time. It is better to process the smaller biopsy tissue separately from the larger tissue to have proper dehydration
Cracked and folded tissue section	Excessive dehydration	The block is soaked with a wet gauze piece before sectioning
Irregular staining of haematoxylin and eosin stain	Dehydration process is suboptimum	Change the processing program, and give adequate time for dehydration
Brittle tissue after processing	Excessive blood in tissue	Apply a gauze piece soaked with warm water on the surface of the block before sectioning

The major disadvantages of manual processing include inconvenient for processing and time taken procedure.

2.7.3 Microwave Processing

Microwave processing in histopathology reduces the time of processing significantly [2]. It is suitable for small number of delicate tissues. The microwave oven usually has:

1. System to control the temperature
2. System to control the time duration of particular temperature
3. Proper exhaust to remove the toxic gas

The microwave processing may be used for all the steps of processing.

Tissue processing for electron microscopy: See Chap. 26.

Troubleshooting in processing is highlighted in Table 2.3.

References

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2. Leong AS, Leong FJ. Strategies for laboratory cost containment and for pathologist shortage: centralised pathology laboratories with microwave-stimulated histoprocessing and telepathology. Pathology. 2005;37(1):5–9.

Embedding of Tissue in Histopathology

3

After processing the tissue, the next step is embedding of the tissue to make the block. In the embedding process, the tissue is surrounded in a molten medium by using a mould. Subsequently this medium is solidified to make a block for cutting thin section of tissue.

Aims of embedding: Embedding medium has three important functions:

1. To give support of the tissue
2. To prevent distortion of the tissue during cutting
3. To preserve the tissue for archival use

The choice of the embedding medium: Various media are used for embedding such as paraffin wax, epoxy resin, methacrylate, carbowax, etc. Paraffin wax is the most commonly used embedding medium. The choice of the embedding medium depends on the following factors:

1. *Type of tissue*: The density of the tissue and the embedding medium should be close otherwise tissue may not be sectioned properly, and tissue will be deformed.
2. *Type of microtome*
3. *Type of microscope*

The basic technique of embedding is the same irrespective of the embedding medium.

3.1 Embedding Medium

(a) *Paraffin wax*: As described in the previous chapter, paraffin wax is a solid polycrystalline hydrocarbon. The paraffin wax is sold in the market with different melting point. Paraffin wax with melting points ranging from 56 to 62 °C is used in our laboratory. Paraffin wax is cheaper and easy to use. Little supervision is needed to make block by it.

(b) *Epoxy resin*: Epoxy resin is mainly used in electron microscopy as it provides better resolution and greater details of tissue.

(c) *Acrylic medium*: Methacrylate monomer is miscible with ethanol. In the presence of catalyst (benzoyl peroxide 2%), methacrylate monomer is polymerized and provides a hard and clear block. Methacrylate monomer is available in the market along with hydroquinone which should be removed by treating with weak alkali solution followed by thoroughly washing with water. The presence of water may lead to small bubbles within the block.

(d) *Agar gel*: Agar gel helps in cohesion of friable and fragmented tissue particularly in cytology sample and also endometrial curetting and small endoscopic biopsies. It does not provide good support of the tissue for section cutting. Agar-paraffin wax double embedding is more suitable technique than agar alone.

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(e) *Gelatin*: It is also used in small friable tissues and frozen section containing friable and necrotic tissue. The melting point of gelatin is 35–40 °C, and this low melting point makes it unsuitable for embedding.
(f) *Celloidin medium*: Celloidin is nitrocellulose and was mainly used for embedding hard tissue. Nowadays it is not used in the laboratory.

3.2 Different Types of Mould Used for Block

Leuckhard Embedding Moulds (Fig. 3.1) Leuckhard embedding moulds have two arms. One arm of the L is longer than the other arm. The two L-shaped arms are adjusted to make a convenient size for block. Adequate lubricant such as glycerine is applied to the L arms and metal plate for easy removal of the tissue. The molten wax is poured in the space between two L arms, and then the tissue is placed within the bottom of the liquid wax. The wax is subsequently cooled, and the block with tissue in one surface is removed for microtomy.

Stainless Still Mould Here the mould is made of stainless steel (Fig. 3.2). The base of the mould is flat and well-polished that helps to remove liquid paraffin. The mould can be covered by a plastic ring.

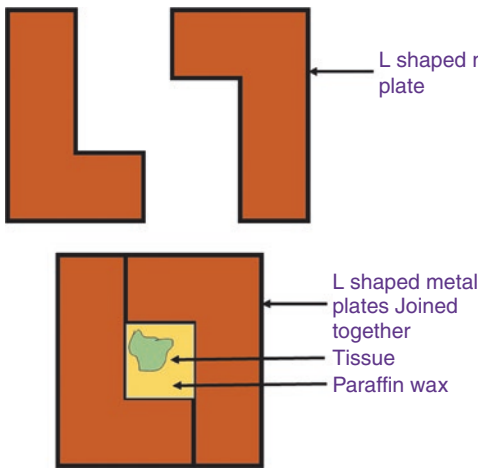

Fig. 3.1 L-shaped Leuckhard embedding moulds

Fig. 3.2 Stainless still mould

Fig. 3.3 Chemical-resistant plastic mould

Plastic Mould Here the mould is made of chemical-resistant plastic (Fig. 3.3).

3.3 Tissue Embedding Method

Tissue embedding method is essentially same for all the types of embedding media.

The following things are needed for tissue embedding:

1. Molten paraffin wax
2. Mould with cover
3. Metal plate (cold plate) to work

Nowadays there are commercially available system with different components together [1]. Tissue-Tek system is the combination of (Fig. 3.4):

1. Dispenser of liquid paraffin in a constant temperature
2. A metal plate to make the tissue block
3. Cold plate

Tissue-Tek system I: The steps of this system are (Fig. 3.5):

- Liquid paraffin is kept at a constant temperature in the dispenser of the system.
- The tissue is put on the lower surface of the mould by forceps. The cutting surface should be faced down.
- Molten paraffin is poured on the metallic mould.
- The mould is covered with peripheral plastic ring on the upper surface.
- Unique tissue number is put in one corner.
- The whole mould is put on the cold plate.
- The metallic mould is detached from the plastic ring, and the block is used for cutting tissue.

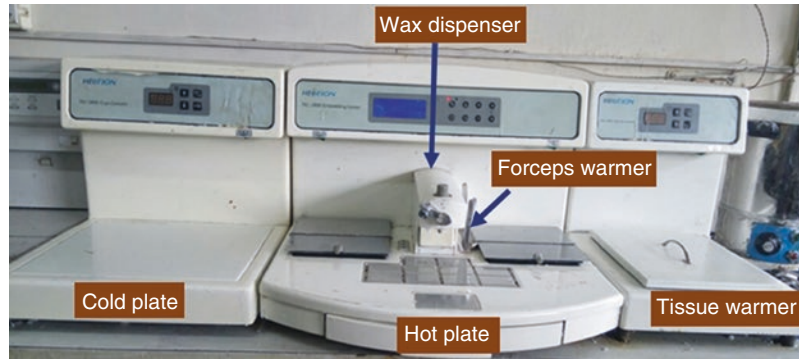

Fig. 3.4 Tissue-Tek system consisting of hot plate, tissue warmer and cold plate

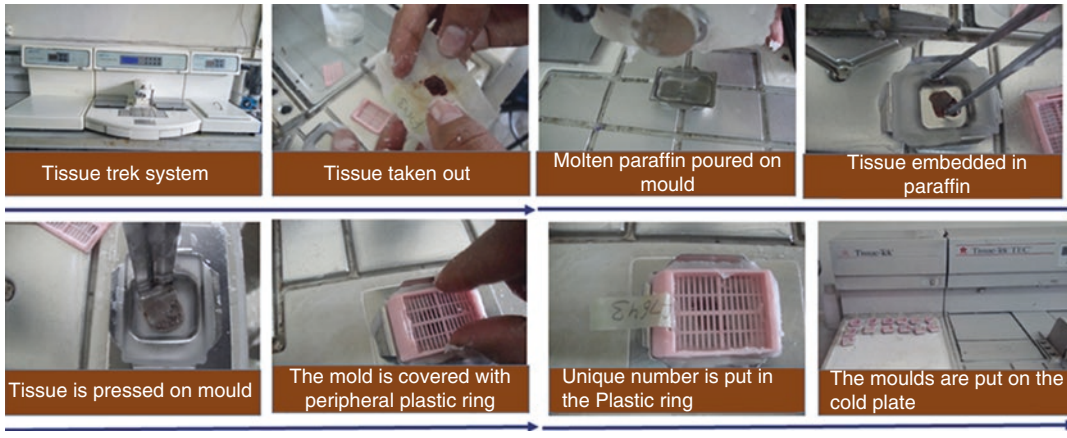

Fig. 3.5 Illustrated view of whole embedding process. At first the Tissue-Tek system is put on. The tissue is taken out from the processing bucket. The molten paraffin is poured in the metal mould. The tissue is embedded with the help of forceps. The tissue is pressed to keep in the molten paraffin. The mould is now covered with the peripheral plastic ring. A unique number is placed in the plastic ring and the mould is now kept on the cold plate to make it firm

Tissue-Tek System II The basic steps of Tissue-Tek II are similar as described above. In this system instead of metallic mould, we use plastic mould to hold the liquid paraffin.

3.4 Tissue Orientation and Embedding

The correct orientation of the tissue is very important for proper cutting and microscopic examination. Tissue is usually placed as flat on the central part of the mould. It should be oriented in such a way so that cutting is easy by knife of the microtome. Some of the tissue needs special care as described below (Fig. 3.6):

1. The tubular tissue (fallopian tube, vas difference, artery, etc.) should be placed in such a manner so that we get a transverse section with all the layers.
2. Tissue with epithelial surface should be placed vertically and right angle to the surface so that we can get all the layers.
3. Multiple section of tissue such as endometrial curetting should be placed all in central position.
4. Linear long tissue should be placed diagonally.
5. Muscle biopsy should be placed both in longitudinal and transverse plane.
6. Long membranous tissue such as amniotic membrane should make as Swiss roll.

3.5 Tissue Marking [2]

The tissue marking by ink is needed for the following purposes:

1. To identify the resection plane or outer margin of the tissue
2. To help in embedding the tissue
3. Any area of interest to identify such as the area of transitional zone in cone biopsy of cervix

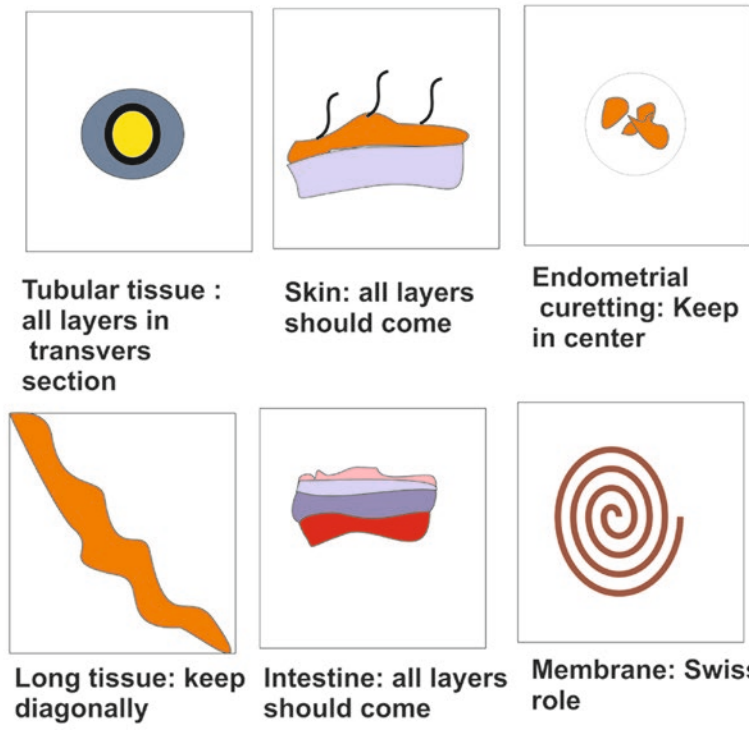

Fig. 3.6 Illustrated view of embedding of different tissue samples

The tissue markers should have the following characteristics features:

- The marker substance should not be dissolved in fixative and tissue processing agents.
- The marker should not penetrate the deeper tissue.
- It should be recognizable in the stained section both microscopically and macroscopically.

The common tissue markers: The common tissue markers include:

- India ink: This is the most commonly used marker in the routine surgical pathology laboratory. It takes 15 min time to mark the tissue.
- Silver nitrate: This is also a good marker. It produces brown-black colour.
- Rose Bengal: For surgical margin stain, 1% Rose Bengal dye is used. It stains within 5 min and provides pink stain.
- *Application of ink*:
- Clean the area and dry the tissue with bloating paper. Completely dry the tissue.
- Apply the dye by a cotton swab.
- Allow some time to dry it.
- Put fixer over the dye. Usually 3% acetic acid or 50% white vinegar is used as fixer.
- Dry the specimen with a sponge.
- Process now.

Troubleshooting in tissue embedding is highlighted in Table 3.1.

Table 3.1 Troubleshooting in tissue embedding

Problem	Cause	Remedies
Fraction of the embedded tissues are cutting	Tissues are embedded in different level	Give pressure after embedding the tissue in the molten wax in mould
Tiny fragments of tissue is seen in the subsequent blocks	Tissue is carried over by forceps	– Clean the forceps every time after embedding – Deal with only one tissue at a time, and so open only one cassette at a time
Epithelium not properly seen	Wrong orientation	Please mark the tissue by ink so that the embedding upper surface can be identified
Tissue is fallen out during microtomy	Air bubbles are entrapped around the tissue	Properly embed the tissue in the molten wax

References

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4 Decalcification of Bony and Hard Tissue for Histopathology Processing

4.1 Introduction

The presence of calcium salt in the tissues makes them very firm to hard and this may damage the knife. Therefore, it is often necessary to remove calcium salt from the tissue and to make it soft for cutting in a microtome. The process of removal calcium salt from the tissue is known as decalcification (Box 4.1).

Aim: The basic aims of decalcification are:

1. Removal of calcium salt from tissue
2. No damage to tissue morphology
3. No significant effect in staining

Calcium-containing tissue: The tissue containing heavy amount of calcium salts are (1) the bone, (2) tooth, (3) pathological tissue such as in tuberculous lymph node, dystrophic calcification, and certain tumours such as teratomas, etc.

Requisites for successful decalcification: The following measures are helpful for successful decalcification:

- *Consistency*: Exact assessment of the consistency of the tissue is required for successful decalcification.
- *Small pieces*: The tissue should be cut in 2–6 mm thick sections because thicker tissue may take longer time to be decalcified.
- *Fixation*: Adequate fixation of the tissue is necessary for proper decalcification.

Box 4.1 Decalcification

Aim: Removal of calcium salt from tissue without damaging the morphology of the tissue

Calcium-containing tissue: (1) bone, (2) tooth, and (3) pathological calcification in tissue

Requisites for successful decalcification:

- Small tissue
- Adequate fixation
- Consistency
- Adequate volume of decalcifying agent
- Suitable choice of the decalcifying agent
- Exact end point detection

Factors controlling the rate of decalcification:

- Facilitates decalcification
- Higher concentration of the decalcifying agent
- Higher temperature
- Agitation of the solution
- Thin tissue
- Low density

Methods of decalcification

- Acid decalcification
- Ion-exchange resin

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Box 4.1 (continued)

- Electrical ionization
- Chelating solution
- Surface decalcification

End point determination of decalcification

- Radiographic examination
- Chemical test
- Physical test

- *Washing*: The fixed tissue should be washed thoroughly before decalcification.
- *Choice of decalcifying agent*: Suitable choice of the decalcifying agent is required.
- *Volume*: Optimum volume of the decalcifying agent is a prerequisite for proper decalcification.
- *End point detection*: The end point of the decalcification should be determined correctly.

4.1.1 Factors Controlling the Rate of Decalcification

- *Concentration*: The increased concentration of the decalcifying agent increases the rate of decalcification.
- *Temperature*: Increased temperature fastens the decalcification rate.
- *Density of bone*: Hard bone takes longer time to be decalcified.
- *Agitation*: Mild agitation of the decalcifying solution increases the rate.
- *Thickness of tissue*: Thinner tissue is quickly decalcified.

4.2 The Methods of Decalcification [1]

1. Acid decalcification
2. Ion-exchange resin
3. Electrical ionization
4. Chelating solution
5. Surface decalcification

Acid Decalcification This is the commonest method of decalcification in routine laboratory process. Acid makes the soluble calcium salt, and thereby calcium is removed from the tissue.

The strong acids:

- Hydrochloric acid
- Nitric acid

Weak acids:

- Formic acid
- Trichloroacetic acid

Strong Acid The strong acids are used in 5–10% concentration. They are rapid in action. However, careful attention is needed to prevent tissue damage. Neutralizer is also used to prevent any tissue distortion.

Aqueous Nitric Acid This is rapid in action. It does not impair staining if the end point is not crossed.

Preparation:

Nitric acid	5 ml
Distilled water	100 ml

Advantages:

1. Rapid in action
2. Good nuclear stain

Precaution: Nitric acid may give yellow colour to the tissue that can be removed by urea.

Nitric acid formaldehyde (10%)

Nitric acid	10 ml
Formalin	10 ml
Distilled water	80 ml

Advantages:

1. Rapid action.
2. Good nuclear stain.
3. Less chance of tissue damage and swelling.
4. Long-time washing by water is not needed.

Von Ebner's fluid

Saturated solution of sodium chloride: 175 g
Hydrochloric acid (concentrated): 15 ml
Distilled water: make it up to 1000 ml

Advantages:

1. Rapid action
2. Ideal decalcifying agent for the tooth

Disadvantage:

1. Nuclear staining is not good.

Perenyi's fluid

Nitric acid (10%)	40 ml
Chromic acid (0.5%)	30 ml
Absolute alcohol	30 ml

Advantages:

1. Provides excellent result
2. Softens the fibrous tissue
3. Cellular morphology well-preserved

Disadvantages:

1. Slower in action.
2. End point detection is difficult.

Weak acids

Gooding and Stewart solution

Formic acid	5 ml
Formalin (40% formaldehyde)	5 ml
Distilled water	90 ml

Advantage:

1. Decalcifying agent of choice in routine laboratory process

Disadvantages:

1. Slow acting and takes many days for decalcification.
2. Increased concentration of formic acid may enhance its capacity.

Trichloroacetic acid

10% Formal saline	95 ml
Trichloroacetic acid	5 g

Advantages:

1. Good for small biopsies
2. Good nuclear stain

Disadvantages:

1. Not good for hard bony tissue
2. Slower in action and takes 4–5 days

Table 4.1 highlights the advantages and disadvantages of various acid decalcifies.

4.3 Chelating Agents

Chelating agents are organic substances that adsorb metals. *Ethylenediaminetetraacetic acid (EDTA)*: EDTA is the most commonly chelating

Table 4.1 Acid decalcifying agents

Chemicals	Concentration	Advantages	Disadvantages
Aqueous nitric acid	5%	1. Rapid in action 2. Good nuclear stain	Gives yellow colour to the tissue
Nitric acid formaldehyde	10%	1. Rapid action: 1–3 days 2. Less chance of tissue damage and swelling 3. Long-time washing by water is not needed	Not a very good nuclear stain
Hydrochloric acid	8%	1. Rapid action 2. Ideal decalcifying agent for the tooth	Nuclear staining is not very good
Trichloroacetic acid	5 g in 95 ml formal saline	1. Good for small biopsies 2. Good nuclear stain	1. Not good for hard bony tissue 2. Slower in action and takes 4–5 days
Formic acid	5%	Decalcifying agent of choice in routine laboratory process	1. Slow acting and takes many days for decalcification

agents in the routine laboratory decalcification (Box 4.2). It binds with calcium of the hydroxyapatite crystals and forms a non-ionized soluble complex. The action of EDTA is slow and gentle, and it may take several weeks to remove calcium from the tissue. Therefore EDTA is not a suitable decalcification agent for dense bone or urgent removal of calcium. The main advantage of EDTA is the preservation of morphology and to maintain the tissue for various other techniques for research purpose. The action of EDTA is pH dependant and it works best in pH 7–7.6.

EDTA solution

EDTA	5.5 g
Formalin	100 ml
Distilled water	900 ml

Advantages:

1. It gives best morphological preservation of tissue.
2. Various other laboratory tests can be done on the tissue such as immunohistochemistry, fluorescent in situ hybridization technique, etc.
3. It is very good for bone marrow trephine biopsy as glycogen is preserved in the tissue.

Disadvantages:

1. Very slow process.
2. Maintenance of pH around 7 is necessary.
3. Thin tissue is needed.

Box 4.2 Ethylenediaminetetraacetic Acid (EDTA)

- Most commonly used
- Chelating agents
- Mode of action: Binds with calcium of the hydroxyapatite crystals to form a non-ionized-soluble complex
- Slow and gentle in action

Advantages:

- Morphological preservation of tissue
- Suitable to do various other laboratory tests
- Very good for bone marrow trephine biopsy

Disadvantages:

- Very slow process.
- Maintenance of pH around 7 is necessary.
- Thin tissue is needed.

4.3.1 Other Procedures of Decalcification

The other uncommon procedures for decalcification include:

- *Ion-exchange resin method*: In this technique an ion-exchange resin (sulfonated polystyrene resin) is used along with an organic acid as decalcifying fluid. It also produces faster decalcification with preservation of morphological details of the tissue.
- *Electrolysis method*: In this process electrolysis of the tissue is done in a solution of hydrochloric acid and formic acid. Calcium from the tissue moves to the cathode plate. This is a very rapid method of decalcification and takes only a few hours to decalcify the bone. However there is a risk of tissue damage in this technique.

4.4 Surface Decalcification

In case of surface decalcification, the surface layer of paraffin blocks is inverted in 1% hydrochloric acid (HCl) for 1 h. The exposed top 30 µm tissue of the paraffin block is decalcified. The block should be washed thoroughly before cutting. Only the first few paraffin sections are expected to be free from calcium.

4.5 End Point Determination of Decalcification

The end point of decalcification can be detected by:

1. Radiographic examination
2. Chemical test
3. Physical test

1. *Radiographic examination*: X-ray examination of the tissue is the most accurate technique to detect the end point of decalcification. However, this is a costly procedure, and the pre-decalcification radiograph is also needed to assess the extent of decalcification.
2. *Chemical test*: This test is done to assess the presence of calcium in the decalcifying solution in two successive times. The chemical test is applied when weak acid solution (e.g. formic acid) is used.

Chemical solution

Stock solution	
Ammonium hydroxide stock solution	
Ammonium hydroxide (28%)	5 ml
Distilled water	95 ml
Ammonium oxalate stock solution	
Ammonium oxalate	5 ml
Distilled water	95 ml

Before use equal volume of both the stock solution is mixed.

Method

- Now for the chemical test, 5 ml of the decalcifying agent from the container containing the tissue is withdrawn.
- Mix the decalcifying agent with 5 ml of ammonium hydroxide and ammonium oxalate mixture solution.
- The mixture is kept overnight.
- Any precipitation is noted.
- The presence of precipitation (calcium oxalate) indicates that the decalcifying agent contains calcium and decalcification is not completed.

3. *Physical test*: This is a crude test and it does not accurately detect the end point of decalcification. The tissue is bent or a pin is introduced within the tissue. In case of adequate decalcification, it is expected that the tissue will be soft and could be bent easily. Pin also should penetrate easily within the tissue. The major disadvantage of physical test is the tissue damage by making a hole or by bending it.

Reference

1. Skinner RA, Hickmon SG, Lumpkin CK, et al. Decalcified bone: twenty years of successful specimen management. J Histotechnol. 1997;20:267–77.

5 Tissue Microtomy: Principle and Procedure

5.1 Introduction

After embedding the tissue and preparing the block, the next step is microtomy. The word "microtomy" is originated from the Greek language. *Mikros* means small and *temnein* means to cut. So the word "microtomy" means to cut the tissue in thin sections. For successful microscopic examination, it is necessary to have thin sections of the tissue by microtomy.

5.2 Microtomes

It is the main instrument by which we cut the embedded tissue in the paraffin block as thin section. The different types of microtomes in the traditional histology laboratory are:

- Rotary microtome
- Rocking microtome
- Base sledge microtome
- Sliding microtome
- Cryomicrotome
- Ultramicrotome
- Laser microtome

(a) *Rotary microtome* (Fig. 5.1): This is the most commonly used microtome in routine laboratory. The cutting blade is kept in horizontal position, and the block containing tissue moves up and down with the help of rotatory handle attached with the microtome. In each 360° rotation of the wheel handle, the block moves down followed by up, and the tissue is cut as thin ribbon. This microtome has the option to be semiautomated or automated with the adjustment and control of the movement of the block and angle of the knife.

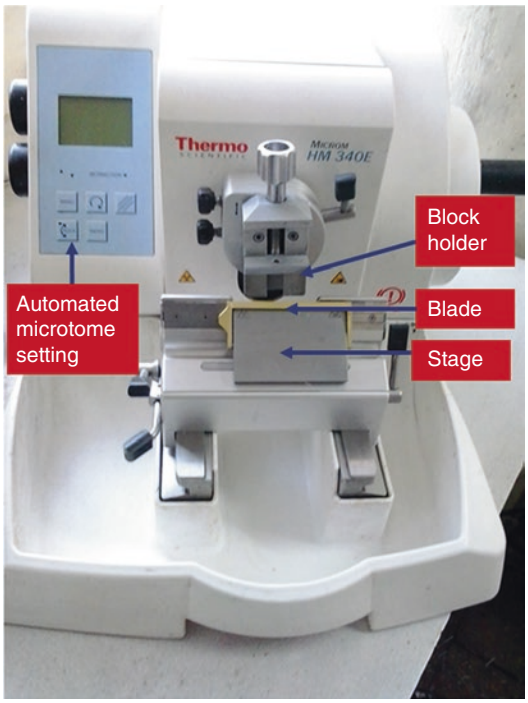

Fig. 5.1 Semiautomated rotary microtome

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Advantages:

1. Good-quality 2–3-μm-thin section is possible.
2. Heavy and stable automated rotary microtome reduces health hazard and gives the best-quality section.
3. Good tissue ribbon production.
4. Easy-to-cut various types of tissue: firm, fragile, small biopsy, etc.

Disadvantages:

1. Expensive.
2. Unsuitable to cut large block.
3. Knife faces up and so may be dangerous to the technical staff.

(b) *Rocking microtome*: The rocking microtome is also known as Cambridge rocking microtome. The word "rocking" is used as there is a rocking action of the microtome like arm movement. In this type of microtome, the knife is static, and the block of tissue moves in a rocking motion (arc-like movement of the block). This is one of the oldest designs of the microtome. The microtome can cut thin section with ribbons and is ideal for serial section. The sections are slightly curved in this microtome.

Advantages:

1. Thin section
2. Easy to operate
3. Low-cost instrument and reliable

Disadvantages:

1. Tissue is curved and the microtome does not provide flat section.
2. As the microtome is of light weight so vibration may occur.

(c) *Base sledge microtome*: In sledge microtome the block is fixed in a static position within a steel carriage. The knife slides to and fro over the top of the block. This microtome is the best for large tissue sample or the hard tissue. The tissue sections are usually thick (more than 10 μm) in base sledge microtome.

Advantages:

1. Hard tissue can be cut.
2. Large tissue sample can be cut.
3. The best microtome for ophthalmology and large neuropathology section.

Disadvantages:

1. Difficult to get thin section.
2. Large slides are required.

(d) *Sliding microtome*: In this microtome the knife is static, and the block moves horizontally over the knife.

Advantages:

1. Large sections can be cut.
2. Mainly used for celloidin-embedded tissue.
3. Simpler design and easy maintenance.
4. Brain sections can be cut better by this type of microtome.

Disadvantages:

1. The knife may glide in case of hard tissue and may jump.
2. Long knives are difficult to sharpen.

(e) *Cryomicrotome*: This type of microtome is used for the cutting tissue for frozen sample (Fig. 5.2). The sample is made hard in liquid nitrogen and then cut by the microtome in the chamber that contains liquid nitrogen.

Advantages:

1. To get rapid section for rapid diagnosis
2. To study nerve biopsy
3. To study enzyme histochemistry

Disadvantages:

1. It needs continuous supervision to maintain the temperature.
2. Freezing artefact is often seen.
3. Very expensive instrument.
4. Fixed tissue is very difficult to cut.

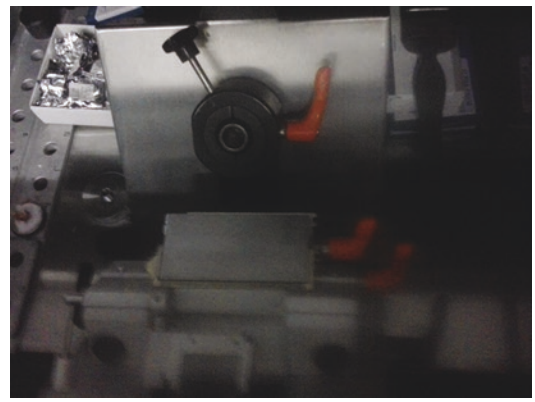

Fig. 5.2 Cryomicrotome used in frozen section

(f) *Ultramicrotome*: Ultramicrotome is used to cut ultrathin sections for transmission electron microscopy. Sections are cut between 40 and 100 nanomicron thickness with the help of glass knife or diamond knife. The tissue is at first trimmed to make small block of 1 × 1 mm size, and then the section is cut by this ultramicrotome with the help of optical microscope. The cut section is allowed to float on the water hold by a boat and then finally picked up on a metal grid.

(g) *Laser microtome*: In this ultramodern microtome, the laser beam is used to cut the biological section without any processing or embedding the material. Infrared laser beam with ultrashort pulse duration is applied, and therefore almost no heat is generated, and the tissue is cut without any thermal effect.

5.2.1 Microtome Knife

The microtome knife is important to cut uniform and thin section of tissue (Box 5.1). These are made of steel. Various types of knife profiles are available for different types of microtomes. The most commonly used knife profile is Profile C or wedge profile. The various types of microtome knife include (Fig. 5.3):

1. *Plano concave (Profile A)*: One side of the knife is plain and the other side is concave. Originally these knives were used for cutting celloidin-embedded tissue. This is a very sharp knife and is used for cutting soft tissues. However, presently these knives are less frequently used.
2. *Biconcave (Profile B)*: The knife is concave on both sides. The knife was used for rocking microtome. The concavity of the knife is often difficult to identify. This is a less rigid type of knife and often vibrates during cutting.
3. *Wedge (Profile C)*: This knife is plain on both sides. This is the most widely used knife for routine microtomy and it is compatible with the different type of microtome. This type of knife is also easy to sharpen.
4. *Tool edge (Profile D)*: The knife resembles chisel used in wood working. Both sides of the knife are plain; however the cutting edge is

Box 5.1 Microtome Knives

Types based on shape:

- Plano concave (Profile A):
  - Very sharp knife and is used for cutting soft tissues
- Biconcave (Profile B):
  - Used for rocking microtome
  - Vibrates during cutting
- Wedge (Profile C):
  - Most commonly used
- Tool edge (Profile D):
  - Used for hard tissue

Types based on disposability:

- Permanent
- Disposable
  - Low-profile blade: To cut small biopsy
  - High-profile blade: To cut firm to hard tissue

Materials used in knife:
Conventional knife: Steel
Diamond knife: Made of diamond and used to cut epoxy resin blocks

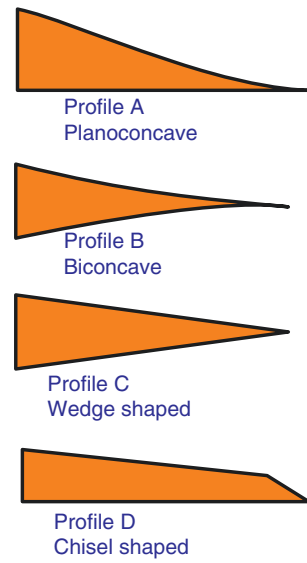

Fig. 5.3 Different types of microtomy knives based on shape. Profile C is the most commonly used knife

steep. The tool edge knife is mainly used to cut the hard tissue such as decalcified bone. The knife is difficult to sharpen and is not recommended presently.

5.2.2 Disposable Knife

Nowadays disposable blade is used in many laboratories to save time to sharpen. Two types of disposable blades are available:

1. *Low-profile blade*: These blades are used to cut small biopsy or soft large tissue.
2. *High-profile blade*: These are used to cut firm to relatively hard tissue such as the uterus, heart, etc.

Advantages:
1. Easy to replace within seconds.
2. No need to sharpen.
3. The overall cost of disposable blade system is low as there is no need of any knife sharpener or abrasive powder to sharpen the knife.

Disadvantages:
1. They are not very rigid like ordinary knife, and therefore vibration effect may be seen.

5.2.3 Materials Used in Knife

Conventional knife: The conventional microtome knifes are made of very high-quality carbon or steel material that are usually tempered from the edge to inside one third of the width. The desirable hardness of the knife is in between 400 and 900 Vickers hardness scale.

The diamond knife: This is a costly knife and is used to cut epoxy resin blocks in electron microscopy. Special care is needed to sharpen this knife.

Glass knife: This is used for ultramicrotomy to cut very hard tissue. The cutting edge of the knife is parallel to one surface of the glass.

5.2.4 Angles of Knife

The various angles formed by the knife include (Fig. 5.4):

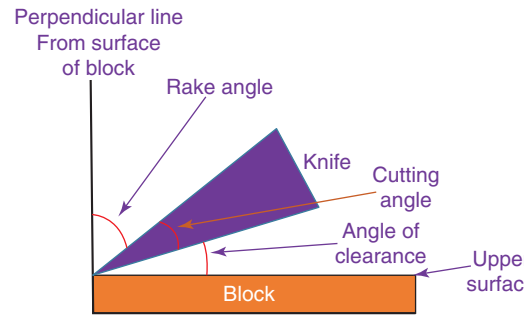

Fig. 5.4 Schematic diagram of angles of knife

Rake angle: It is the angle between the upper bevel of the knife and the perpendicular line drawn from the surface of the block. Increased rake angle makes the section cutting easier.

Angle of clearance: It is the angle between the lower bevel of the knife and the surface of the block. It is usually around 5°. The angle of clearance is related with the friction between the block and knife. Lower the angle of clearance, the less will be the compression on the block.

Bevel angle: This is the angle in between the two planes of the knife.

Cutting edge: This is the straight line formed by the intersecting of the two planes of the bevel.

5.3 Microtome Knife Sharpening

Sharpening of the microtome knife is needed to get good sections. The following methods help in sharpening of knife:

5.3.1 Manual Method

Honing Abrasive grindings are used to sharpen the knife. Stone can be used to sharpen the knife. Light oil can be used to lubricate the stone before use.

Iron oxide, aluminium oxide and silicon carbide are usually used as abrasive. The handle of the knife is hold by one hand, and the other hand holds the front end of the knife. The knife is pushed forward and diagonally over the slab

several times. Same procedure is followed in other surface of the knife also.

Stropping It helps to polish the cutting edge of the knife that is already sharped by honing and also to remove any "burr" formed during honing. Strop is made of leather and it should be free from any dust or grit.

Automatic Knife Sharpener Presently automatic knife sharpener is available in the market. The knife is placed horizontally on the surface of the rotating plate made of glass or copper coated with abrasive agents.

5.3.2 Factors Involved in Cutting

1. *Temperature*: Lowering the temperature facilitates section cutting.
2. *Angle of rake*: Higher rake angle helps in smooth flow of ribbons. Lower rake angle is used for hard tissue.
3. *Consistency of tissue*: Soft tissue is cut at a slow rate than the hard tissue.

5.4 Sectioning the Paraffin Block

The following instruments are essential for section cutting:

1. Microtome with blade
2. Water bath
3. Paraffin block with embedded tissue to cut
4. Ice tray
5. A blunt forceps or camel brush
6. Slide rack with slides

Water Bath (Floatation Chamber) Water bath is used to float the tissue after cutting (Fig. 5.5). The temperature of the water bath is usually controlled automatically by a thermostat. The temperature of water in the water bath should be 10 °C below the melting point of the embedded paraffin wax and is usually kept in 40–50 °C. It is necessary to prevent formation of any air bubbles within the water bath. For adequate floating of the tissue, one can add a few drops of alcohol or little amount of detergent. This reduces the surface tension of the water and tissue floats smoothly.

Blunt Forceps and Camel Hair Brush Blunt forceps helps to manipulate the floating tissue section (Fig. 5.6). Camel hair brush is used to clean the blade.

Slide Rack with Clean Glass Slides The clean slides are kept in the slide rack. The slides can be already labelled by diamond pencil or on the frosted side by lead pencil. Alternatively this can be marked after lifting the tissue section.

Adhesive In case of routine section and staining, no adhesive is required. However, in certain situations we use cell adhesive material such as:

Fig. 5.5 Water bath used in microtomy. The constant temperature (usually 40–50 °C) is maintained in the water bath. This is usually 5–10 °C lower than the melting point of the paraffin

Fig. 5.6 Blunt forceps and brush used in section cutting

1. Brain sections
2. Decalcified tissue
3. Using strong alkali at the time of staining

The most commonly used adhesives include:

- *Mayer's egg albumin and glycerol*: This is prepared by mixing
  - White part of egg: 100 ml.
  - Glycerol: 100 ml.
  - Homogenize the mixture thoroughly, and filter it by gauze piece. Add few crystals of thymol to prevent bacterial growth.
- *Poly-l-lysine*: This is a good adhesive and does not produce any background staining. The slides are coated with poly-L-lysine diluted by distilled water (1:10) before using.
- *3-Aminopropyltriethoxysilane (ACEP)*: The slides are dipped in the dilute ACEP solution in acetone (2%) and then dried before using.
- *Permanent positively charged slides*: Here the slide is tempered in such a way that the slide surface is always positively charged. It is easier to lift the tissue section with these slides. The positively charged slides are also excellent for lifting frozen section tissues.

5.4.1 Steps of Tissue Sectioning (Fig. 5.7)

1. *Trimming the tissue*: Trimming of the tissue is needed to expose the tissue piece within the paraffin wax for cutting. The block is fixed in the chuck of the microtome, and the paraffin is cut till the tissue is fully exposed. Adequate caution should be taken not to overcut tissue as this may produce various artefactual changes.
2. *Cooling the block*: After the initial trimming, the blocks are kept for cooling for 15–20 min. This will help to maintain the same consistency of the paraffin and tissue. This helps in easy cutting.

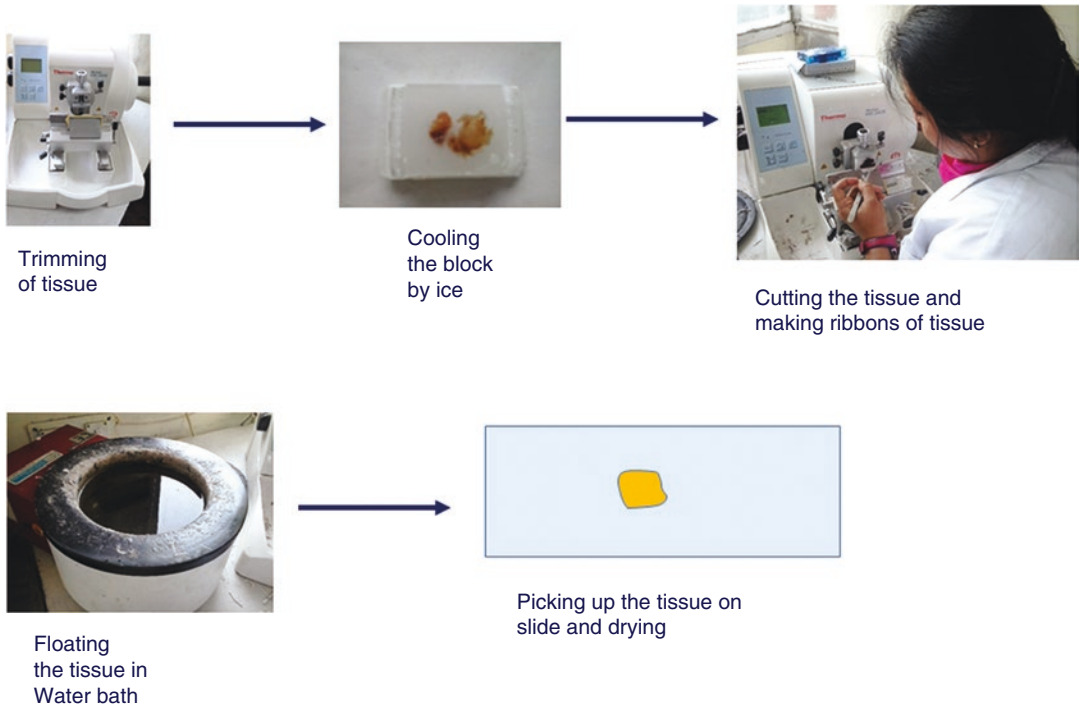

Fig. 5.7 Different steps of section cutting are highlighted here. At first the tissue is trimmed. Then the block is cooled in ice. The block is placed in the microtome, and the angle of clearance is adjusted to 5 °C. The tissue is gently cut, and the tissue ribbon is placed with the help of brush on the water bath. Then the tissue is picked up by a putting a glass slide perpendicularly in front of it, and then when the tissue is touched, the slide is withdrawn vertically

3. *Cutting proper*: The block is fixed in the chuck of the microtome. The cutting surface of the block should be parallel to the knife. The angle of clearance should be only 2–5° to have good section (Fig. 5.8). The tissue in the block is cut by gentle, smooth and slow stroke. The ribbon-like tissue sections are produced. The tip of the ribbon is held by forceps, and the end part of the ribbon is removed from the knife edge by brush. In case of any difficulty to get the flat section, the cutting surface should be gently warmed by warm water.

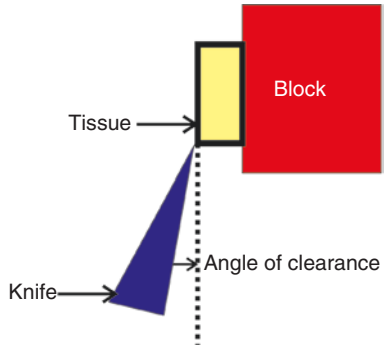

Fig. 5.8 The schematic figure of the tissue and the position of knife making the correct angle of clearance which is 5°

4. *Floating the ribbon*: The ribbon of the tissue is floated in the water bath, and this makes the tissue flat and removes any wrinkling of the tissue. With the help of the forceps, the individual sections are separated from each other. As mentioned before, the temperature of the water bath should be constantly maintained below the melting point of the paraffin wax. In case of temperature variation in the bath, the air bubbles may be formed that may rupture the tissue.
5. *Picking up the tissue*: The slide is placed vertically within the water bath in front of the tissue, and when the tissue is touched, the slide is withdrawn vertically from the water. The tissue pickup process must be gentle and smooth. To prevent any mix-up, the water bath should be cleaned immediately after cutting each block.
6. *Drying the section*: The slide containing the picked-up section is kept in slide rack. The slides are now kept in hot oven to get dry. The temperature of the oven should be slightly more than the melting point of the paraffin.

Various problems may occur in tissue sectioning (Figs. 5.9, 5.10, 5.11, 5.12 and 5.13) [1]. These are enumerated below in Table 5.1.

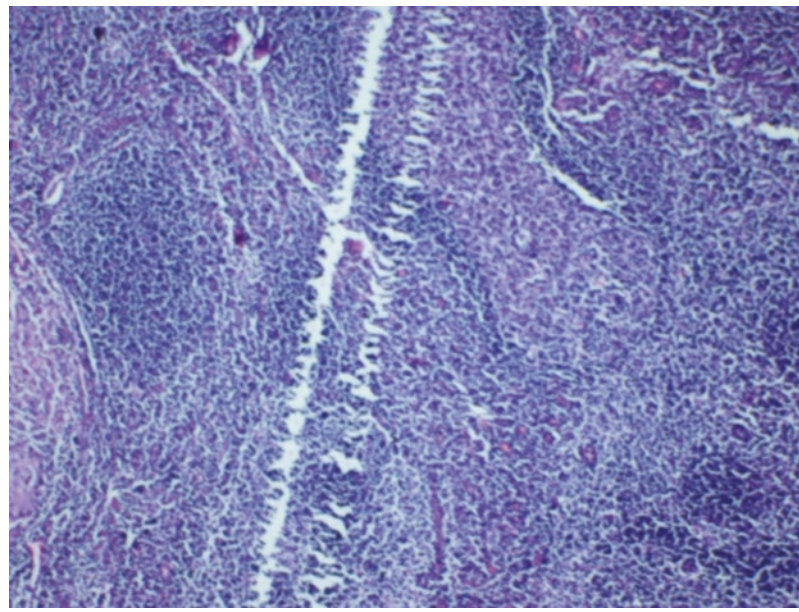

Fig. 5.9 Tear in the tissue due to uneven cutting edge of the knife

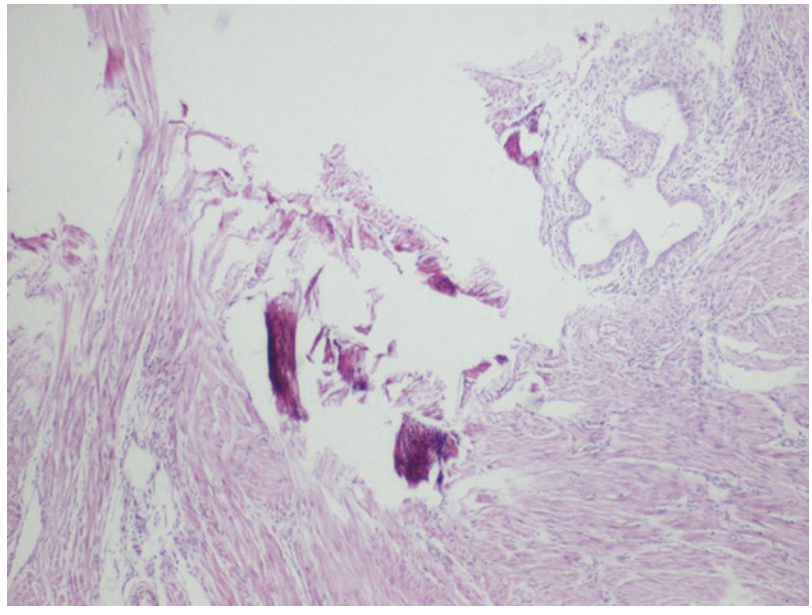

Fig. 5.10 Large hole in the tissue due to the bad processing

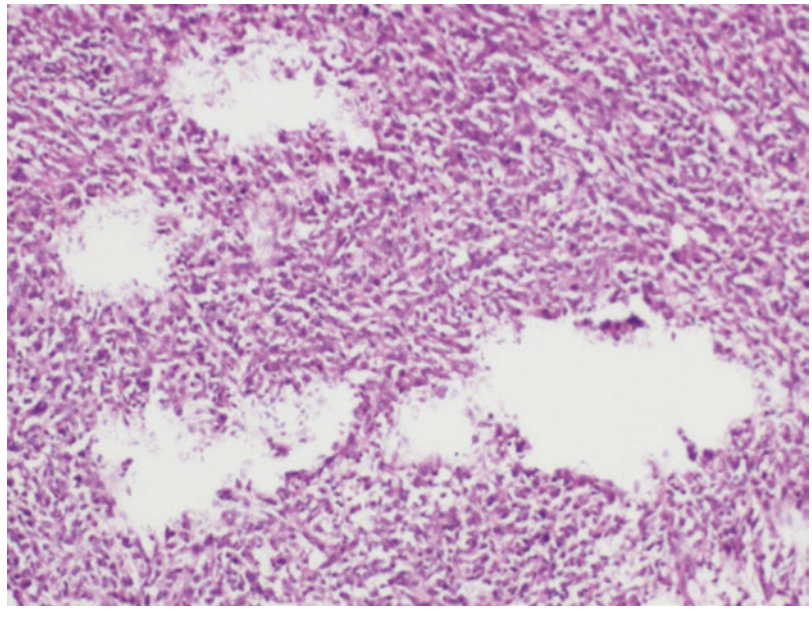

Fig. 5.11 Multiple air bubbles have ruptured the tissue. The air bubbles are produced due to uncontrolled temperature in the water bath. Note the characteristic multiple irregular tear of the tissue

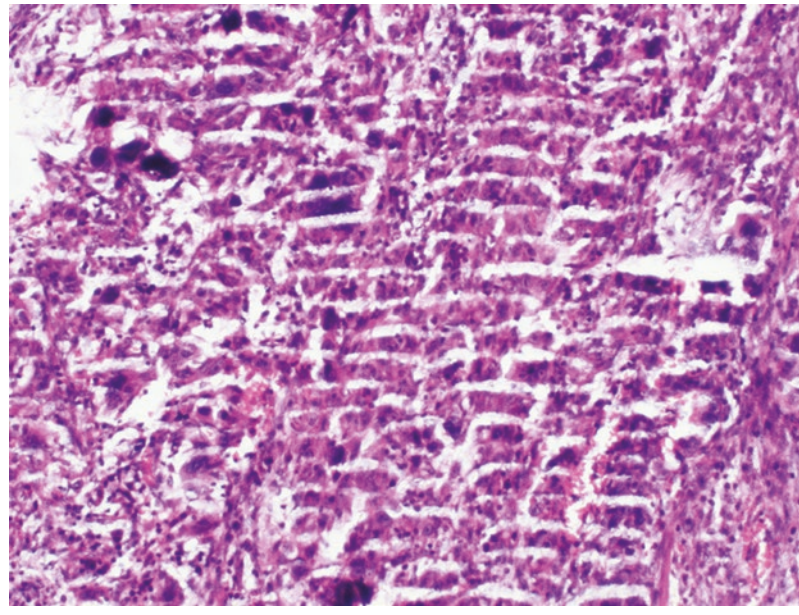

Fig. 5.12 Freezing artefact of the tissue due to immediate putting the tissue from the refrigerator to formalin. Note the regular spaces in between the tissues due to melting of ice crystals

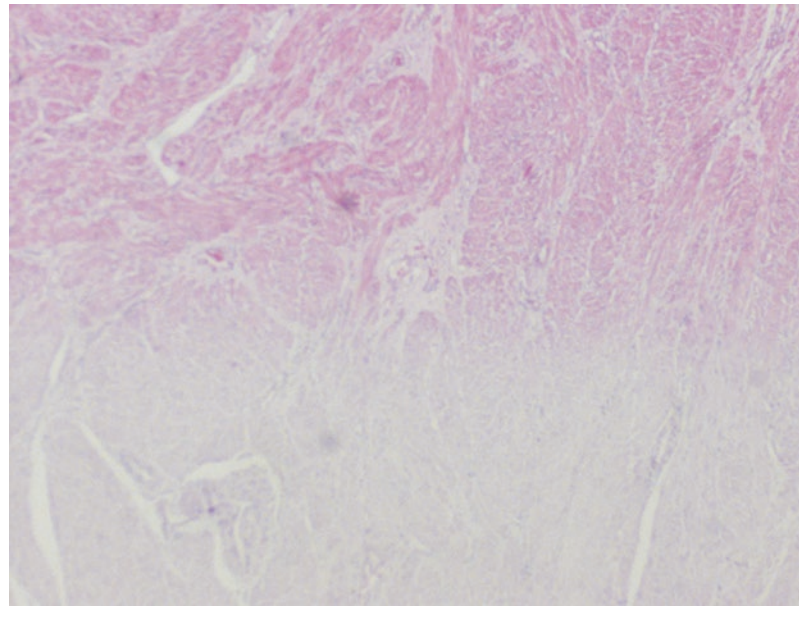

Fig. 5.13 Uneven staining pattern due to poor deparaffinization of the tissue

Table 5.1 Troubleshooting in tissue sectioning

Faults	Source of faults	Solution
Ribbons not formed: the tissue is twisted and curled or sticks to the knife	• Paraffin is hard • Clearance angle is incorrect: small • Dirty knife • Too cold or hot room temperature	• Select low melting point paraffin • Correct the knife alignment • Clean the blade • Adjust the room temperature
Ribbons are curved: the ribbons are not straight and are coiled	• Edges of the block are not parallel: the sides of the block and the knife edge are not in right angle • Surface of the block is uneven • Blade is not sharp • The paraffin is too much	• The block holder should be aligned properly • Trimming is needed • Sharpen the knife or replace the blade • Trim and remove the excessive paraffin
Ribbons are excessively compressed and wrinkled	• Block is warm • Dull blade/knife • Paraffin is soft and sticky • Knife clearance is not optimum and is too small • Rotation of the microtome is clumsy	• Cool the block • Replace the blade or sharpen the knife • Cool the block and then try to cut; otherwise the paraffin should be replaced with higher melting point • Increase the knife clearance • Needs consistent and gentle rotation of the wheel
Thick and thin section (chatter)	• Knife or block is loose • Blunt knife • The clearance angle is very small • Paraffin is soft and sticks to the knife	• Tighten the knife clamps or the chucks • Sharpen the knife or change the blade • Adjust the clearance angle • Clean the knife and try to remove the attached paraffin
Section attaches to the block	• Static electricity is generated in the knife or ribbon • The clearance angle is defective	• Apply ionizer to remove the static charge, and/or clean the blades, holder, etc. with alcohol • Increase the clearance angle
Tear or scratches in the section	• Defect in the blade: a nick or jagged knife edge • Dirt in the knife • Sharp particle in the tissue • Sharp particle in the paraffin	• Hone the knife/use other part of knife/change the blade • Clean the blade • Decalcify the tissue • Try to remove sharp particle by scalpel
Tissue disintegrate in the water bath	• Too hot water • Faulty and incomplete processing	• Temperature should be lowered down • Reprocess the tissue
Large holes in the tissue	• The underprocessed tissue ruptures when comes in contact with warm water	• Reprocess the tissue

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Frozen Section: Principle and Procedure

6

6.1 Introduction

The frozen section is the rapid tissue section by cooling the tissue with the help of cryostat to give immediate report of the tissue sample. This is especially needed in large hospital to diagnose the lesion or extent of the lesion at the time of operation. The cryostat is the instrument that has the arrangement to freeze the tissue and also to cut the frozen tissue for microscopic section.

6.2 Indications of Frozen Sections

The frozen section is used mainly for immediate diagnosis of the lesion for management and to know the extent of the lesion [1–3] (Box 6.1). It is also helpful to do enzyme immunochemistry and immunofluorescence study. At times, frozen section tissue is used for the demonstration of fat and carbohydrate in the tissue sample.

Box 6.1 Indications of Frozen Section

- Rapid diagnosis of the lesion for intraoperative management
- To know the extent of the lesion
- To do enzyme immunocytochemistry
- To do immunofluorescence study
- To stain lipid and certain carbohydrate in the tissue

6.3 The Principle of Frozen Section

The rapid freezing of the tissue sample converts the water into ice. The firm ice within the tissue acts as embedding media to cut the tissue. Lowering the temperature makes the tissue more firm, whereas increasing temperature makes the tissue softer.

Cryostat Machine Proper (Fig. 6.1)

Temperature range in the machine: The cryostat machine has the usual temperature range from 0 °C to −35 °C. The most of the tissue is sectioned properly between −15 °C and −25 °C. The water-containing tissues can be sectioned in higher temperature, and fat-containing tissue needs much lower temperature to cut.

Rotary microtome (Fig. 6.2): Rotary microtome is placed inside the cabinet of the cryostat. Here the knife is fixed and the tissue is moved with the help of a rotary wheel.

Tissue shelf (Fig. 6.2): Just in one side of the microtome, there is a tissue shelf to keep the tissue. In this place the samples are kept for freezing. Usually the temperature of tissue shelf is lower than the overall cabinet temperature.

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https://doi.org/10.1007/978-981-10-8252-8_6

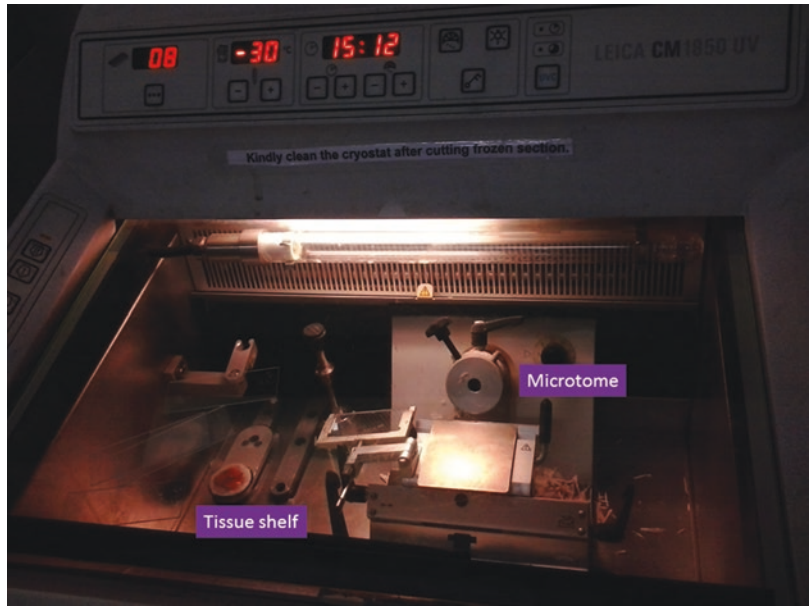

Fig. 6.1 Cryostat machine with its parts

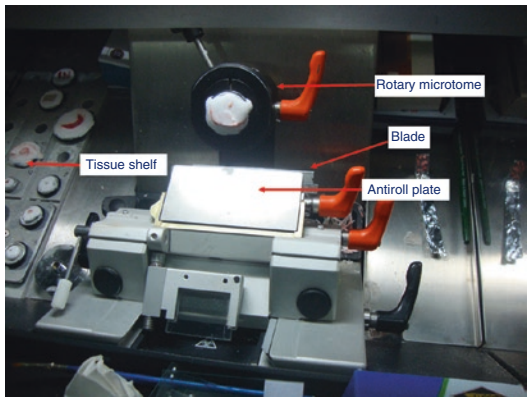

Fig. 6.2 Microtome, blade, antiroll plate and tissue shelves are shown

Place to keep the brush and knife holder: Just in front of the microtome machine, there remains a small place to keep the brush and knife holder.

Knife or blade: Nowadays, low- or high-profile disposable blades are used. The blade should be proper fixed to the holder to get an even pressure in the whole length. Alternatively Profile C steel blade is also used. The angle of the knife is kept in between 5° and 7°.

Antiroll plate (Fig. 6.2): Just in front of the knife, there is an antiroll plate that prevents the rolling of the cut tissue. It is usually a glass plate within a metal frame. The undersurface of the plate has free space, and there is a gap between the knife and the plate.

Alternating to antiroll plate, a cool sable hair brush can be used to get unrolled tissue.

Specimen holder: The specimen holder or chuck is supplied by the manufacturers in different sizes and shapes. Usually these are round metal structures.

Embedding medium: This medium is used to hold the tissue over the chuck. Presently optimum cutting temperature (OCT) compound is used as embedding medium. The OCT is made of water-soluble glycols and resin.

6.4 Cryostat Sectioning

The process of the cryostat sectioning needs the following steps.

1. *Grossing and cutting the specimen* (Box 6.2): The cutting surface of the tissue should be smooth. The following steps in grossing of the tissue are mandatory for accurate reporting:
   - *Identify the tissue sample of the patient and the requisition form*: This is the first and foremost part of the frozen tissue grossing.

Box 6.2 Grossing for Frozen Section Tissue

- *Identify the tissue sample of the patient.*
- *Clinical information*: provides possible differential diagnosis.
- *Tissue appearance*: colour, texture, nodule, any suture.
- *Anatomy of the tissue*: identify the resection planes and margins.
- *Colour the resection planes and margins*.
- Section cutting:
  - Use sharp blade.
  - First cut the most important area.
  - Give gentle pressure and avoid too much pressure.
- Cytology preparation: if needed make
  - Imprint smear
  - Scrape smear
  - Crushed smear

- *Salient clinical information*: The essential clinical information is very helpful as it guides the pathologist to reach the possible differential diagnosis.
- *Tissue appearance*: The gross appearance of the tissue such as colour, texture, consistency and any suture to mark the anatomical position.
- *Resection margin*: It is very important to identify the resection margins of the tumour. The resection planes and margins should be inked thoroughly. The different colours of ink can be used for medial and lateral margin identification.

Cutting the tissue: The tissue should be fresh without any fixative. The tissue should be preferably dry, and it should not be wrapped in gauze piece. Any suture, staple or sharp hard structure should be removed from the tissue sample. Now the tissue is cut into small pieces as it facilitates freezing. Take multiple sections of the tissue to understand the main pathology and to minimize the error. Use a new sharp scalpel blade, and first cut the most important area that needs microscopic examination. It is preferable to use gentle stroke of the scalpel rather than too much pressure.

Cytology of the tissue: At times the imprint of the tissue on the slide provides good morphological details such as lymphoma of the lymph node. Similarly crushing of tissue also provides excellent morphological details such as in case of tissue of the brain tumour.

1. *Tissue embedding in the mould* (Fig. 6.3): The small piece of the tissue is kept in the centre of the mould, and then the OCT is poured over it in excess. Then the tissue holder or chuck is firmly placed over the tissue with overflown OCT.
2. *Tissue loading in the frozen section chamber*: The tissue is now placed in the frozen section chamber, and cold spray can be used to make the process faster.
3. *Loading the blade*: The cutting knife or blade is now loaded and the proper alignment is done.
4. *Trimming the tissue*: The loss of normal or natural colour to whitish colour indicates that the tissue is frozen. The frozen tissue in the tissue holder is now placed in the holder of the microtome. The block is trimmed to remove the excess OCT and to get the smooth tissue surface for sectioning.
5. *Sectioning* (Fig. 6.3): The tissue is now cut gently and is spread over the antiroll plate with the help of a brush. The brush should be cooled. The tip of the tissue is guided by the brush.
6. *Section lifting*: The glass slide of normal room temperature is pressed firmly over the tissue section, and normally the tissue sticks immediately.
7. *Fixation*: The tissue should be immediately fixed in methanol for 1 min or 95% ethanol for few seconds. Rapid fixation within few seconds is mandatory. In case of delayed fixation, the cells are swollen, and the cytoplasmic margin may be ruptured giving hazy appearance of the margin of the cells.

Troubleshooting in frozen section: Various problems may arise during the cutting of frozen section tissue. This has been highlighted in Table 6.1.

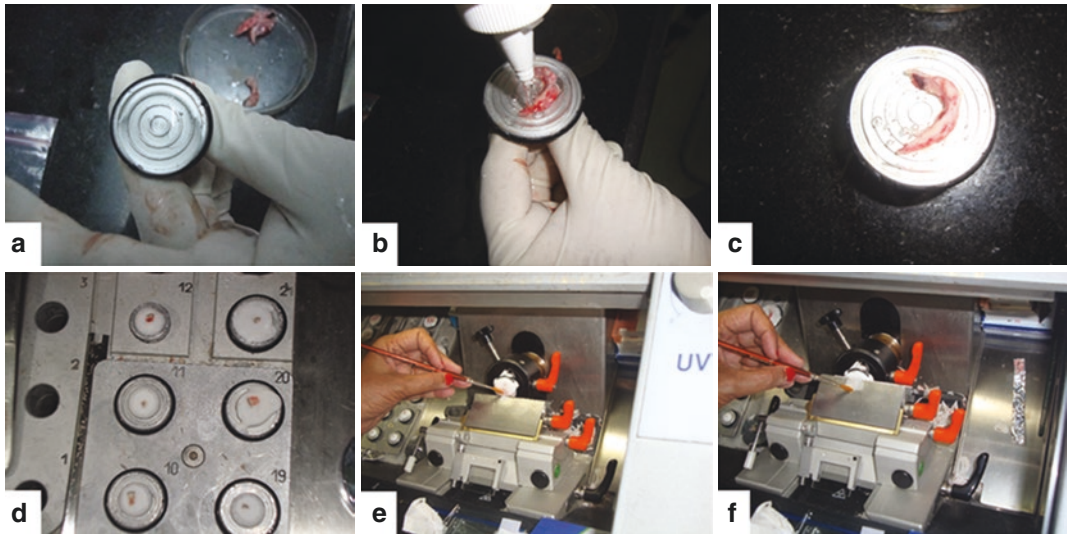

Fig. 6.3 Cryostat processing: (**a**) mould is covered with OCT, (**b**) the tissue is now put on the block, (**c**) OCT is flooded over the tissue, (**d**) the tissue now is put in the cooling chamber, (**e**) the brush guides the tip of the tissue, (**f**) the tissue section is gently spread over the antiroll plate and later picked up by touching a glass slide

Table 6.1 Troubleshooting in frozen section

Problems	Cause	Solution
Freezing artefact	Formation of ice crystal within the tissue. Water-containing tissue shows more such artefact	• Freeze the tissue rapidly, i.e. snap freezing
		• The tissue specimen should not be in saline solution before freezing
Uneven tissue embedding	The surface of the tissue is uneven, and the vital information may be lost	• Make tissue even at the cutting surface before freezing
Block is loosen during chucking	The chuck may be too cold when the tissue is placed on it	• Take the tissue out and reattach it on a clean chuck which is not too cold
Tissue crumpled	The tissue in the block is warm or too cold	• Make the block of tissue in the optimum temperature: −15 °C to −20 °C
Chattering artefact	The temperature of the block is too cold, and the tissue becomes hard. The blade will cut the tissue thick and thin in regular interval	• Bring the block in optimum temperature. Pressing the cut surface of the block by gloved finger may make the block warmer
Thin stripe in tissue	The perpendicular tear in the tissue is due to nicks on the blade	• Replace the blade by a sharper one
Widely striped tissue and also tearing of the tissue	This may happen if the tissue is sticking with the blade	• Clean the blade or replace with a new one

Fixation: Immediate dip in 95% ethyl alcohol for a few seconds fixes the tissue.

6.5 Staining

We commonly use haematoxylin and eosin (H&E) and toluidine blue stain.

6.5.1 H&E Staining

- Rinse the slide in tap water.
- Put in haematoxylin for 1 min.
- Rinse in tap water for 5 s.
- Rinse in Scott's tap water for 5 s for bluing.
- Dip in eosin for 20 s.
- Rapidly rinse in tap water.

- 95% ethanol for 10 s.
- 100% ethanol for 10 s.
- 100% ethanol for 10 s.
- Dip in xylene for 20 s.
- Mount by DPX.

6.5.2 Toluidine Blue Stain

This is a very simple stain and takes only a few seconds. The drops of toluidine blue stain are put on the section, and the coverslip is put on the section. The slide is now ready to see. The histopathologist feels more comfortable in H&E stain than this unfamiliar toluidine blue stain.

6.6 Factors Affecting the Good-Quality Section

The common factors responsible for the good-quality smear include:

- *Temperature*: when the temperature falls, water within the tissue becomes frozen and gives the tissue hard consistency. The optimal temperature of frozen tissue is in between −15 °C and −25 °C. Warm tissue remains soft and sections crumple. On the other hand, the overcooled tissue becomes very hard and brittle and produces again bad-quality crumpled section. Moreover the hard tissue may cause "chattering" artefact and also thick and thin sections. Different tissue contains variable amount of fat and water. The consistency of different tissue varies, and therefore the optimum temperature to cool the tissue varies considerably. Table 6.2 shows the optimum temperature of different organs to have good frozen section.
- *Tissue consistency*: other than the optimum cooling temperature, the consistency of tissue has significant effect on cutting such as:
  (i) Fatty tissue: It is difficult to cut the fatty tissue in frozen section. Fat may smear on the knife and may make problem in cutting.
  (ii) Collagenous tissue: The firm collagenous tissue is difficult to cut.
  (iii) Necrotic tissue: Soft necrotic tissue may create considerable problem as they may fall from the slide making hole in the section. It is preferable to take only viable tissue for frozen section.
  (iv) Bony hard tissue: Hard tissue like bone or cartilage may damage the blade significantly. In this situation a new section can be processed, or new blade can be used.
- *Tissue size*: The size of the tissue sample should be small as the larger tissue takes much longer time to freeze.

Table 6.2 Optimum temperature for frozen section

Tissue	Optimum temperature
Brain, liver, spleen	−7 °C to −10 °C
Rectum, uterus, adrenal, muscle, skin	−12 °C to −15 °C
Heart, lung, intestine, pancreas, ovary, cervix, prostate	−16 °C to −20 °C
Bone marrow, breast	−20 °C to −25 °C

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Staining Principle and General Procedure of Staining of the Tissue

7

7.1 Introduction

The tissue section is colourless because the fixed protein has the same refractive index as that of glass. We use dyes that have specific affinity with the different tissue proteins and colour them differently. This helps us to understand the morphology of tissue.

7.1.1 Dyes Used for Staining

The dye may be natural or synthetic. The natural dye is extracted from plants and animals. Nowadays natural dye is rarely used except haematoxylin and carmine. The majority of the synthetic dye is petroleum derivatives. All these synthetic dyes have a central benzene ring (Fig. 7.1). The benzene has chemical formula C_6H_6, and it is in a ring form which is very flexible. Benzene is colourless; however if certain chemical group is inserted into the benzene ring, then it will impart colour, such as when two H atoms in benzene ring are replaced by two O atoms, then the compound quinone ($C_6H_4O_2$) is formed which is a chromogen. This grouping in the benzene ring that imparts colour is known as chromophore, and the chemical compound formed by the grouping is known as chromogen.

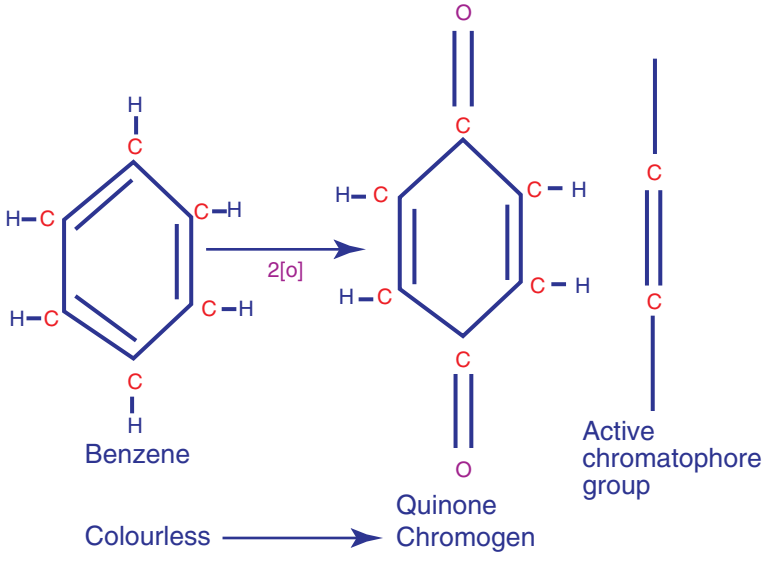

Fig. 7.1 Benzene ring. Benzene itself is colourless. When two H atoms in benzene ring were replaced by two O atoms, then the compound quinone ($C_6H_4O_2$) is formed which is a chromogen

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https://doi.org/10.1007/978-981-10-8252-8_7

How Dye Produces Colour The visible light has the range of wavelengths between 400 and 650 nm. The white light contains all the seven colours (VIBGYOR: violet, indigo, blue, green, yellow, orange and red) with the wavelength in between 400 and 650 nm. A chromogenic dye absorbs the light of particular wavelength of the white light representing a specific colour and emits the light containing the rest of the colour. Therefore we see the particular colour of the dye,such as the dye that absorbs red light will be visible as green coloured in the naked eye.

The dye is the most elementary component of staining of a tissue. In general dye has two components: chromophore and auxochrome part (Fig. 7.2):

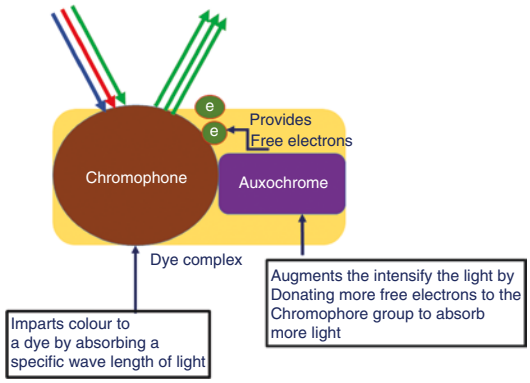

Fig. 7.2 Schematic diagram showing colour generation by dye. The chromophore group absorbs light and imparts colour to the stain. The auxochrome group donates more electrons to the chromophore group and helps to absorb light of longer wavelength in the visible range

- *Chromophore groups*: The chromophore group absorbs light and imparts colour to the stain. These groups have many free electrons that absorb the ultraviolet rays of light which are not in the visible range.
- *Auxochrome part*: This part of the dye helps to intensify the light. It is an ionizing group that also helps to stick the stain with the tissue. The auxochrome group augments more free electrons in the chromophore groups. The increased number of electrons in the system helps to absorb light of longer wavelength in the visible range.

7.1.2 Types of Dye

The dye can be classified on the basis of electrical charge (Table 7.1):

(a) Anionic dye or acid dye
(b) Cationic dye or basic dye
(c) Neutral dye
(d) Ligand dye (chelating)

(a) *Anionic dye or acid dye*: These dyes carry negative charge (coloured anions), and in an electrical field, they migrate towards anode. The dye is mostly of low molecular weight and soluble in water. Most of the dyes have two or more anionic groups which makes them soluble in water. These acid dyes combine with the tissues that carry positive charges, and those tissues are called as "acidophilic".

Example: Eosin is one of the most commonly used anionic (acid) dyes.

Table 7.1 Types of dye based on electrical charge

Types of dye	Charge of the dye	Tissue to bind	Example
Anionic dye or acid dye	Negatively charged	• Cytoplasmic proteins • Collagen	Eosin
Cationic dye or basic dye	Positively charged	• Nucleic acid • Epithelial mucin	Methyl green, ethyl green and Alcian blue
Neutral dye	Contain both acid and basic dye	• Both nucleus and cytoplasm	Giemsa
Ligand or chelating dye	Weak acid so anionic and negatively charged	• Nucleus and cytoplasm	• Al ligand with haematoxylin: Harris haematoxylin and Mayer's haematoxylin • Fe ligand with haematein: iron haematoxylin

(b) *Cationic dye or basic dye*: Cationic dye carries a positive charge (coloured cation) and move towards cathode in an electrical field. The cationic dyes are mostly soluble in ethanol. These are basic dyes and combine with tissues that carry negative charge. This negatively charged tissue combining with basic dye is called as "basophilic" tissue.

Example: The examples of basic dye are methyl green, ethyl green and Alcian blue.

(c) *Neutral dye*: These are the compound dyes that contain both acid and basic dyes in combination. In aqueous solution, the acid dye and basic dye exchange electrons and combine together to precipitate in the tissue. Romanowsky-Giemsa staining is the best example of the compound dye that undergoes electron transfer process. Romanowsky-Giemsa contains azure B and eosin Y. The azure B dye is the electron acceptor, and eosin Y is electron donor.

Example: Giemsa

(d) *Ligand or chelating dye*: Ligand dye is the complex compound that consists of dye and a metal ion. They are also known as metallochrome. They are usually weak acids. Haematein is the oxidized haematoxylin, and it is used as a combination of aluminium (Al) ions or iron (Fe) ions. The metal-ion complex has surplus charge that increases the solubility in water and makes the dye insoluble in alcohol, and therefore dehydration due to ethanol does not occur during staining.

Al^{3+}-Haematein It is a type of ligand dye where the metal aluminium (Al) is combined with haematein. It is used in Harris haematoxylin and Mayer's haematoxylin. As Al^{3+}-haematein is insoluble in ethanol, it can be used along with other anionic dyes, and both dyes are retained even after dehydration with alcohol.

Iron Haematein (Fe^{2+}-Haematein) Iron haematin is the ligand dye that consists of Fe and haematein. The dye is made by combining iron salt and haematoxylin. Fe binds with haematoxylin molecule by oxidizing it to haematein. Iron haematein is used for staining myelin and elastic fibres.

7.1.3 Types of Dye Based on Chemical Structures and Chromophore Groups

1. *Azo dye*: These dyes contain –N═N– chromophore group. Majority of the azo dyes are anionic (acid) dye. Example: orange G and Congo red
2. *Thiazine dye*: Thiazine dye contains –C–N═C– and –C–S═C chromophore group. Example: toluidine blue and methylene blue
3. *Triphenylmethanes*: Triphenylmethanes contain ═N– chromophore group. Example: methyl violet, light green and malachite green
4. *Azin dye*: This group of dye contains C–O═C and C–N═C chromophore. Example: Celestine blue and Nile blue sulphate
5. *Diphenylmethanes*: They contain –NH chromophore group. Example: auramine
6. *Xanthene dyes*: Example—eosin, Rose Bengal and phloxines
7. *Oxazine dyes*: They contain C–O═C chromophore group. Example: cresyl violet and Celestine blue
8. *Acridine dyes*: The dyes of this group are derived from acridine. Example: acridine orange
9. *Anthraquinone dyes*: These dyes are derived from anthraquinone. Example: carminic acid

7.2 Mechanisms and Theory of Staining

The staining is the combination of a coloured substance (dye) with the tissue that retains the dye after washing. The staining is primarily a chemical reaction between the dye and the tissue. The following chemical reactions are involved between the dye and tissue components (Box 7.1) [1, 2]:

1. Electrostatic bond
2. van der Waals attractions
3. Hydrogen bond
4. Covalent bond
5. Hydrophobic bond
6. Dye aggregations

Box 7.1 Mechanism of Staining

- *Electrostatic bond*
  - Coulombic force
  - The electrostatic bond between two oppositely charged particles: dye and tissue
- *van der Waals attractions: weak non-coulombic force*
  - Dipole-dipole interaction
  - Dipole-induced dipole interaction
  - Dispersion or London force
- *Hydrogen bond*
  - Weak bond
  - Type of covalent bond
- *Covalent bond*
  - The two electrically neutral atoms share electron with each other.
- *Hydrophobic bond*
  - Misnomer as in standard chemistry, there is no such bonding
  - Probably a type of van der Waals force
- *Dye aggregations*
  - Dye molecules aggregate in solution and then penetrate into tissue.

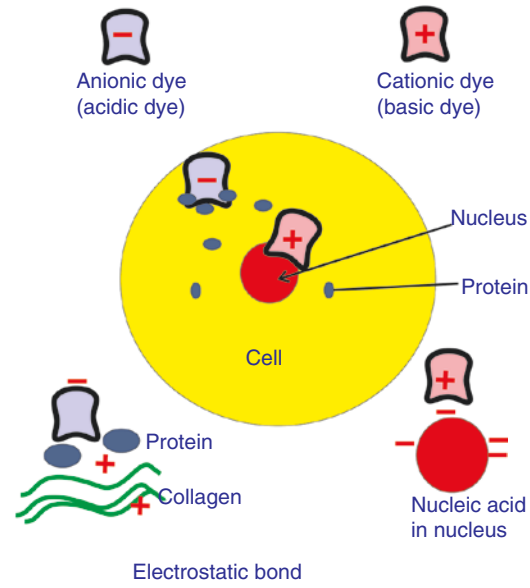

Fig. 7.3 Schematic diagram showing interaction of anionic and cationic dye with the oppositely charged tissue components. Acid or anionic dye binds with the cytoplasmic protein and collagen, whereas the basic or cationic dye binds with nucleic acid of the nucleus

1. *Electrostatic bond*: The electrostatic bond occurs between two oppositely charged particles, and coulombic forces work between the particles. The oppositely charged dye binds with the tissue (Fig. 7.3). For example, acid dye containing the anionic (negative charged) chromogen binds with the acidophilic tissue containing positive charge. Similarly the basic dye having a positively charged cationic chromogen binds with the basophilic tissue containing negative charge.

   The dye is the combination of chromogen and auxochrome that are oppositely charged. In the solution, the dye is dissociated into oppositely charged chromogen and auxochrome, such as basic dye which dissociates into cationic chromogen (positive) and anionic auxochrome (negative). Now the cationic positively charged chromogen of the basic dye combines with negatively charged tissue

   Example: Eosin, the acid dye, stains the cytoplasmic proteins.
2. *van der Waals attractions* (Fig. 7.4): This is a type of non-coulombic force. This is the weakest force due to the intermolecular interactions. The strength of this force is only 0.4–4 kJ/mol compared to 20 kJ/mol in ionic bond. When the electrons of an atom concentrate in one pole of the atom, then a dipole is formed. This dipole is just like a magnet having two poles.

   Dipole-dipole interaction: The positive charge of a permanent dipole interacts with other permanent dipoles, and electrostatic interaction occurs.

   Dipole-induced dipole interaction: Similarly a permanent dipole may induce adjacent atom and induces dipole. This permanent dipole may interact with induced dipole.

   Dispersion or London force: Permanent dipole may induce the adjacent atom as

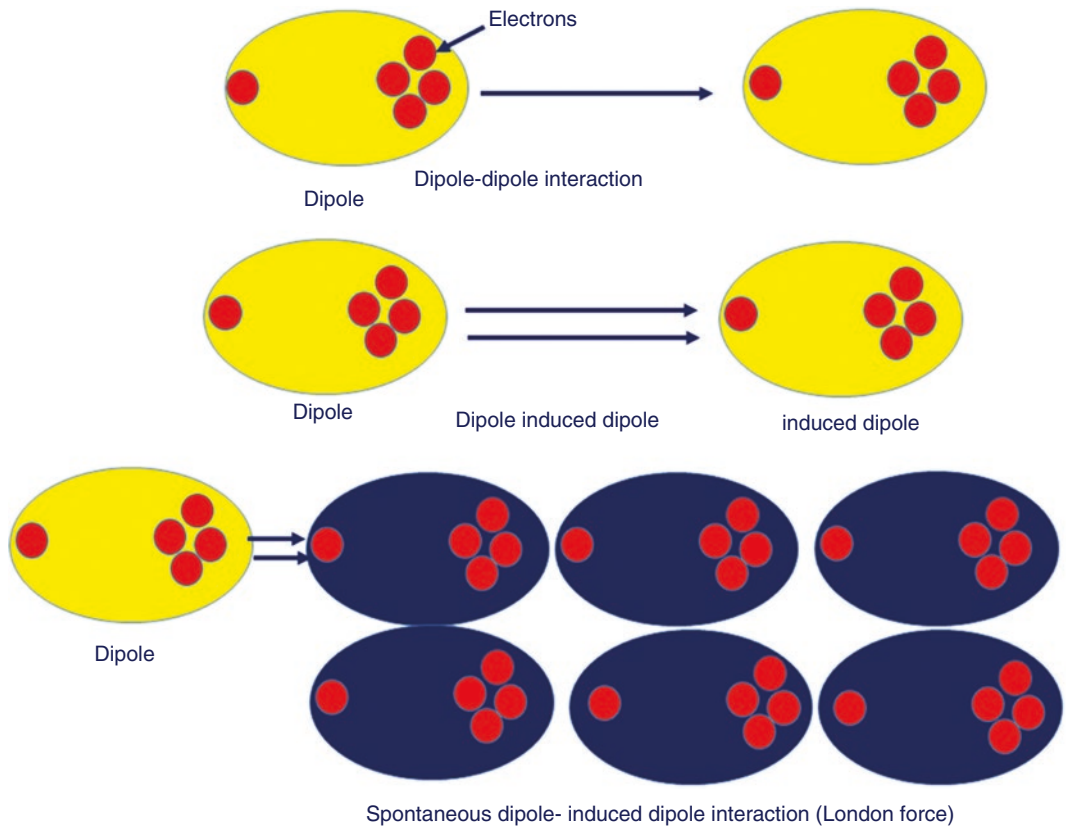

Fig. 7.4 Schematic diagram of van der Waals force. The positive charge of a permanent dipole interacts with other permanent dipoles. In case of dipole-induced dipole interaction, the permanent dipole interacts with induced dipole. In case of London force, the permanent dipole induces the adjacent atom as induced dipole that further induces a chain of induced dipole and forms a large network of tissue with induced dipole interaction

induced dipole. The induced dipoles further induce a chain of induced dipole. In this way a large network of tissue may undergo induced dipole interaction. This is known as dispersion or London force.

Example: elastin stain by Miller's stain and Congo red stain.

3. *Hydrogen bond*: Hydrogen bonding is a weak bond. It is a type of covalent bond that occurs between hydrogen and a strong electronegative atom commonly O, N or F. Water forms hydrogen bond and so competes with stain-tissue bonds. Therefore, hydrogen bonding in dye-tissue less likely occurs in aqueous solution. Example: Best's carmine dye to stain glycogen.
4. *Covalent bond*: In case of covalent bond, the two electrically neutral atoms share electron with each other to satisfy the outer shell's required number. The covalent bond is a stronger bond.

   Example: periodic acid Schiff's staining for glycogen and Feulgen reaction.
5. *Hydrophobic bond*: This is a misnomer as in standard chemistry, there is no such bonding. It is probably a type of van der Waals force. When two hydrophobic molecules interact, then London force, the dispersion type of van der Waals force, interacts. Therefore instead of hydrophobic bonding, the better terminology is probably "hydrophobic interaction" [3].

   Example: staining in aqueous solution and metachromatic staining.
6. *Dye aggregations*: Dye molecules may interact with each other forming dye-dye

interaction. They aggregate in solution and then penetrate into tissue. The dye-dye aggregate increases when the dye concentration is high, the molecular size of the dye is bigger and temperature is low.

7.3 Factors Influencing Staining

Several factors have influence on staining intensity (Box 7.2):

1. Dye affinity to the target tissue specimen
2. Specimen geometry
3. Target concentration
4. Rate of reaction
5. Rate of stain loss

1. *Dye affinity to the target tissue specimen*: The tendency to bind a dye with the target tissue is known as dye affinity. The acidic dye such as eosin binds strongly with acidophilic target, that is, cytoplasmic protein. The acidic dye has very little affinity with basophilic substances. Affinity of the dye to the specific tissue is also influenced by the pH and the presence of inorganic salt concentration of the solvent.
2. *Specimen geometry*: Specimen geometry or topography also influences the staining.
   (a) Thick tissue: If the tissue is thick, then the penetration of dye is difficult, and the central part of the tissue takes poorer stain.
   (b) Surface topography: The surface of the tissue of paraffin section is more even than cryostat section, and so it takes better stain.
   (c) Disturbance of microtopography of tissue: The alcohol is a coagulative fixative that disturbs the topography of the cell and tissue. The shattering effect of alcohol increases the dye penetration rate.
   (d) Inner geometry of tissue: The inner geometry of the tissue may also influence staining such as bone marrow canaliculi which are rapidly stained by Schmorl's thionine stain than the adjacent connective tissue.
3. *Target concentration*: The concentration of the target tissue affects the staining intensity as the more the amount of the target tissue, the more intense will be the staining.
4. *Rate of reaction*: Rate of reaction in the target tissue also influences the staining pattern such as in Feulgen reaction, the short reaction time exposes only a few aldehyde groups producing weak staining pattern.
5. *Rate of stain loss*: Staining pattern is greatly influenced by rate of stain loss. At times the stain loss may be intentional such as differentiation or destaining. The differentiation often removes the excess stain from the cell and thus helps to differentiate the organisms.

Box 7.2 Factors Influencing Staining Intensity

- *Dye affinity to the target tissue specimen*: Acid dye binds with positively charged acidophilic tissue, and basic dye binds with negatively charged basophilic tissue.
- *Specimen geometry*:
  - Thick tissue: Less penetration of dye.
  - Surface topography: More even surface gives better stain.
  - Microtopography of tissue: Alcohol fixation disturbs microtopography.
  - Inner geometry of tissue.
- *Target concentration*: The more the amount of the target tissue, the more intense will be the staining.
- *Rate of reaction*: Short reaction time often decreases stain intensity.
- *Rate of stain loss*: Too much differentiation often removes the stain.

7.3.1 Nomenclature Used Regarding Dye

Colour Index of Dye A certain dye may be called in different names, or the same name may be given to many different dyes. Therefore the simple name of a dye may evoke confusion. To avoid this

confusion, the "society of dyers and colourist" has labelled a specific dye by the unique code known as colour index or colour index (CI) number. The CI number is actually a compendium of dye. With the help of the CI, one can identify the exact dye such as CI 52000 which indicates thionine.

7.4 Metachromasia [4]

The word "meta" means altered, and "chromasia" means colour, so "metachromasia" indicates altered colour. Metachromasia is defined as staining phenomenon when the tissue is stained in different colours from the original dye colour. The metachromatic dye is defined as the alteration of the original colour of the dye without any change of the chemical structure of the dye (Box 7.3).

7.4.1 Metachromatic Dyes

Cationic Dye Majority of the metachromatic dyes are positively charged cationic or basic dyes such as toluidine blue, methylene blue, azure A and B, methyl violet, brilliant cresyl blue, etc.

Anionic Dyes Only a few anionic dyes are metachromatic such as Biebrich scarlet and bromophenol blue. These anionic dyes are weakly metachromatic.

Mechanism of Metachromasia (Fig. 7.5) Glycosaminoglycans of the connective tissue and epithelial mucins and granules of mast cells are negatively charged polyanions. The cationic (positively charged) dye in aqueous solution reacts with the polyanions of the tissues. The binding of the dye molecule with these polyanions of the tissue neutralizes the positively charged dye. The nonpolar aromatic ring of the dye binds with the other dye by van der Waals force. The dye-dye aggregation occurs, and dimer, tetramer and polymer of the dye molecules are formed. Overall dye binding becomes stronger due to van der Waals force. The dye absorbs light of shorter wavelength, and the visible colour of the light emitted from the dye tissue changes. This causes metachromasia or altered colour, such as pyronin Y in the tissue that gives red to orange colour.

Box 7.3 Metachromasia

Metachromasia is a staining phenomenon when the tissue is stained in different colours from the original dye colour.

Metachromatic dyes

Cationic dye: Toluidine blue, methylene blue, azure A and B, methyl violet, brilliant cresyl blue, etc.

Anionic dyes: Biebrich scarlet and bromophenol blue.

Mechanism of metachromasia: The cationic (positively charged) dye in aqueous solution is neutralized with the polyanions of the tissues. Subsequently the nonpolar aromatic ring of the dye binds with the other dye by van der Waals force. The dye absorbs shorter wavelength of light, and the visible colour changes.

Various types of metachromasia:

- Alpha (orthochromatic): Blue colour
- Beta (di- and trimeric form): Purple colour
- Gamma (polymeric form): Red colour

Factors enhancing metachromasia:

- Higher dye concentration
- Low pH
- Decreased temperature
- Aqueous solution

Various Types of Metachromasia (Fig. 7.6) In relation to thiazine dye, three types of metachromatic change may be seen [5]:

- *Alpha (orthochromatic)*: The dye remains in monomeric form and gives blue colour.
- *Beta (di- and trimeric form)*: The dye forms dimeric structure and produces purple colour.

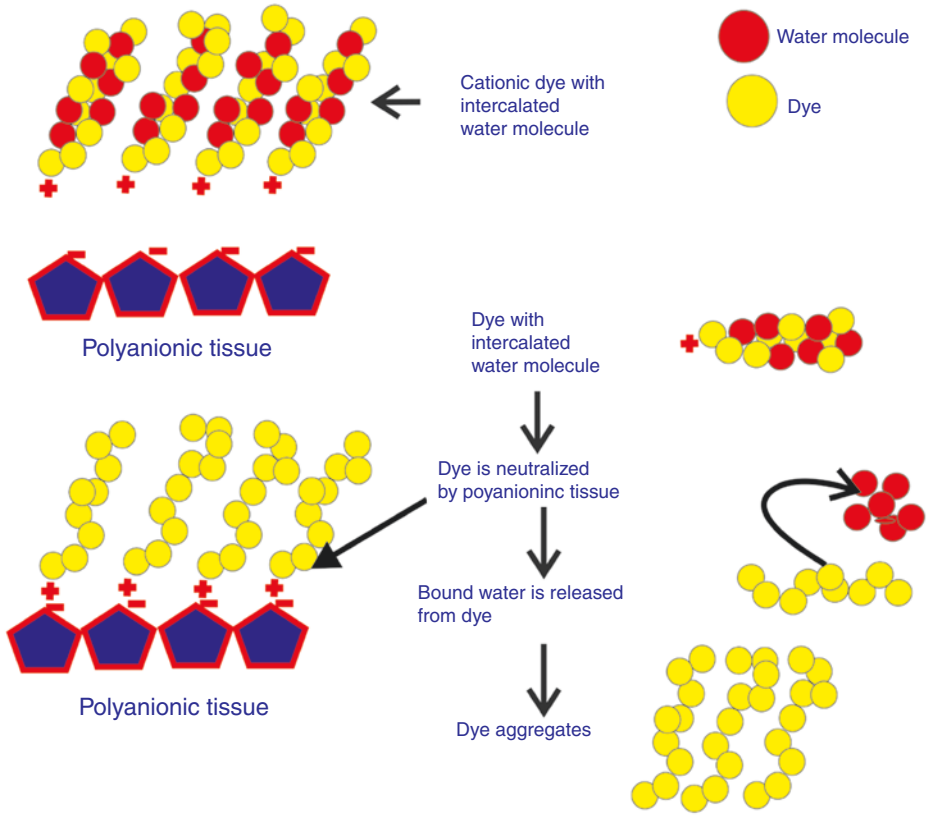

Fig. 7.5 Mechanism of dye aggregation in metachromasia. The cationic dye interacts with the polyanionic tissue, and the bound water of the dye molecule is released. The dye aggregates and the absorption of light changes by the aggregated dye-tissue complex

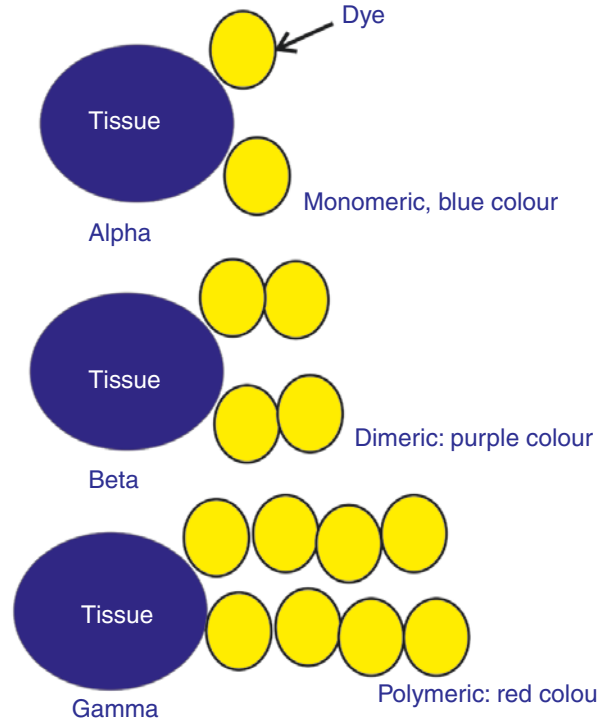

Fig. 7.6 The different types of metachromasia are highlighted in this schematic diagram

- *Gamma (polymeric form)*: The dye is in polymeric form and produces red colour.

The colour changes in metachromasia may not be homogenous. At times the anions in the tissue may be widely distributed. In such cases, there may be purple colouration of tissue due to the mixture of orthochromatic blue and polychromatic red colour.

Factors Influencing Metachromasia The following factors may influence on metachromasia:

1. Dye concentration: High concentration of dye enhances metachromasia.
2. pH: Low pH increases metachromatic effect.
3. Temperature: Decreased temperature augments metachromatic effect.
4. Aqueous solution: Water enhances van der Waals force in between the dye molecules and increases metachromatic effect.

7.5 Progressive and Regressive Staining

Progressive Staining In case of progressive staining, the dye is allowed to react with the tissue until it stains the target structure. In fact, this is a difficult task to supervise, and all other influencing factors should be controlled such as pH of the dye solution, thickness of tissue, concentration of dye, etc. It is always necessary to check the staining at frequent intervals to prevent overstaining or to have light staining.

Regressive Staining Here the tissue is intentionally overstained by dye. The affinity of the different structures of the tissue with dye is variable, and this particular property is exploited to remove the dye from the unwanted part of the tissue. This procedure is also known as *differentiation*. The differentiation is done by using:

(a) Acid in basic dye or base in acid dye such as in Papanicolaou's staining. Haematoxylin is removed from the cytoplasm by using 1% acid alcohol.
(b) Oxidizing agent: The oxidizing agents are used to oxidize the dye and make it a colourless material such as picric acid, potassium permanganate, etc.
(c) Mordant: Here the dye-mordant complex at first binds with the tissue. Subsequently excess mordant is used that attracts the attached dye in the tissue. The mordant thereby removes the excess dye from the tissue.
(d) One dye is replaced by other less affinity dye.

7.6 Mordant

Mordant is the salt and hydroxides of the metals that help in the attachment of dye with the target tissue (Box 7.4). The metal used as mordant is either divalent or trivalent such as aluminium, iron, copper, etc. The mordant binds with the dye by covalent or co-ordinate bonding. This dye and mordant combination is also known as "lake". The mordant-dye combination is basic in action irrespective of the character of the dye. Mordant is insoluble in most of the biological fluid, and

Box 7.4 Mordant

- It is the salt and hydroxides of the metals that help in the attachment of dye with the target tissue.
- It makes the dye strong.
- The mordant binds with the dye by covalent or co-ordinate bonding known as "lake".
- Type
  - Pre-mordanting: The tissue is at first treated with mordant followed by dye.
  - Meta-mordanting: Mordant in combination with dye is used.
  - Post-mordanting: The dye material is applied first followed by mordant.

Example: aluminium and haematoxylin

therefore the staining is not altered even after subsequent treatment of the tissue.

Mordant may be used in three ways:

1. Pre-mordanting: The tissue is at first treated with mordant followed by dye.
2. Meta-mordanting: Mordant in combination with dye is used.
3. Post-mordanting: The dye material is applied first followed by mordant.

Example: Haematoxylin itself is a poor dye. However the combination of mordant such as aluminium and haematoxylin makes a stronger dye.

7.6.1 Accentuators

Accentuators are the group of substances that help to increase the staining intensity of the dye. Accentuators neither form any dye lake nor do they take part in any chemical reaction. The common example of accentuator is using potassium hydroxide in methylene blue solution.

7.7 Staining Procedure

The proper organization of the staining room is mandatory to get a well stained section. The staining room should be well ventilated and well illuminated (Box 7.5).

The workflow of histology/cytology laboratory is fixation, grossing, processing, mounting, microtomy and staining. Therefore the staining room should also be designed accordingly. The staining bench should be facing the window. The bench should be cleaned properly with arrangement of fume remover. There should be at least two supplies of running tap water with sink.

Stains and Equipment The reagents should be kept in the rack with proper arrangement and label (Box 7.6). The list of the reagents should be in the laboratory catalogue. The glass bottle is the best container to store the reagents. The use of amber-coloured bottle is preferable for the dye that reacts with light. Frequently used reagents can be kept in small glass bottle or Coplin jar. A microscope is necessary to check the stain. Automated strainer can stain large batches of slides containing more than 100 slides. Many laboratory prefers manual staining for small batches of slides. In that case, the glass troughs are used. It is preferable to use the series of sequential arrangement of glass troughs for staining. All the troughs should be well covered to prevent evaporation of the reagents particularly alcoholic solution. In addition the laboratory should have ample supply of distilled water.

The Preparation of the Staining Reagent The preparation of staining reagents is one of the most important tasks in any laboratory. Adequate cautions should be taken regarding cleaning glassware, using distilled water instead of tap water, following proper stepwise protocols to make the staining solution and maintaining the concentration of alcohol in alcoholic solution (Box 7.7).

Box 7.5 Staining Room

- Arrange the room according to workflow.
- Do not congest the staining room.
- Clean room and work bench.
- Well ventilated and well illuminated.
- Running water with sink.

Box 7.6 Reagents and equipment

- All reagents in the rack is labelled glass container.
- Maintain the readily available catalogue.
- Amber-coloured bottle for the reagents that needs protection from light.
- The glass troughs well covered.
- Ample supply of distilled water.
- Light microscope.

Box 7.7 Essential precautions during preparation of staining solution

- Clean glassware
- Distilled water instead of tap water
- Alcoholic solution to keep in airtight container
- Stepwise proper protocol to follow
- Fresh solution in case of ammonia solution
- Proper filtration of the staining solution before bacteriological stain
- Silver-containing solution to be kept in dark

7.7.1 Preparation of Buffer Solutions

7.7.1.1 Molar Solution

1M = one molar solution means molecular weight of the compound expressed in gramme dissolved in 1000 ml of water.

0.1M means one-tenth of the molecular weight of the compound in gramme dissolved in 1000 ml water.

7.7.1.2 Citrate Buffer

Citric acid: Dissolve 2.101 g of citric acid in 100 ml of distilled water.

Sodium citrate solution: Dissolve 2.9412 g sodium citrate in 100 ml of distilled water.

Final preparation: Now mix 46.5 ml of citric acid solution with 3.5 ml of sodium citrate solution. The distilled water is mixed in it to make up to 100 ml. This will make 0.1 M citrate buffer. The pH of the solution is adjusted to 2.5 with the help of pH metre by adding 1 N HCl and 5 N NaOH.

7.7.1.3 Phosphate Buffer

Stock A: 0.2M of sodium dihydrogen orthophosphate—Mix 31.2 g of sodium dihydrogen orthophosphate (molecular weight 156) in 1000 ml water.

Stock B: 0.2 m disodium hydrogen orthophosphate—Mix 28.3 g disodium hydrogen orthophosphate (molecular weight 142) in 1000 ml water.

To get pH 6: Mix 438 ml of stock A with 62 ml of stock B, and make up to 1000 ml by distilled water.

7.7.1.4 Tris-HCl Buffer

Stock A: 0.2M Tris.

Mix 2.42 g Tris(hydroxymethyl aminomethane) (of molecular weight 121) in 100 ml water.

Stock B: 0.2M HCl.

Mix 1.7 ml HCl (molecular weight 36.46) in 100 ml distilled water.

To get pH 7.2: Mix 25 ml of stock A with 22.1 ml of stock B, and make up to 100 ml by distilled water.

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8 Haematoxylin and Eosin Stain of the Tissue Section

8.1 Introduction

Haematoxylin is the most commonly used dye in the pathology laboratory [1, 2]. In combination with eosin, this dye is almost indispensable for routine morphological visualization of tissue to every histopathologist. Haematoxylin is a good nuclear stain, and it stains the nuclei bluish black. However, the dye also stains collagenous material, minerals and myelin fibres.

8.2 Haematoxylin

Haematoxylin is extracted from the bark of *Haematoxylum campechianum* tree that is mainly seen in the Campeche state of Mexico. Presently it is also available in West Indies.

Extraction To get haematoxylin, the freshly cut tree of *Haematoxylum campechianum* is chopped in small pieces and is boiled in water. At first the orange-red solution is formed which on cooling turns into a black solution. The compound haematoxylin is then precipitated with the help of urea or ether, and a brownish tan powder is obtained. This is further purified and is sold commercially. The compound haematoxylin has almost no staining capability unless a mordant is used to enhance its staining capability.

Oxidation of Haematoxylin (Fig. 8.1) Haematoxylin has no capability to act as a dye. Its oxidation product haematein is a weak purple-coloured dye. The oxidation of haematoxylin to haematein can be done by two ways:

(a) *Natural*: This is done by exposing the haematoxylin powder in sunlight and air. This is also

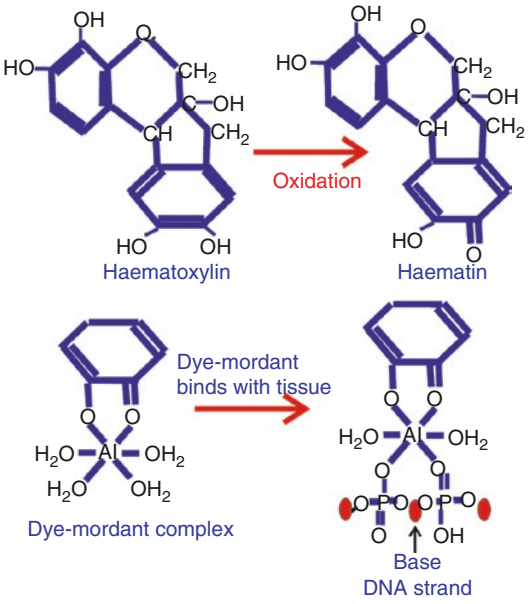

Fig. 8.1 Schematic diagram showing oxidation of haematoxylin and its combination with aluminium

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_8

Table 8.1 Essential types of haematoxylin

Stain type	Mordant used	Application	Time to stain	Comments
Mayer's haematoxylin	Potassium or ammonium alum	Popular nuclear counterstain	5–10 min in progressive stain and 10–20 min in case of regressive stain	
Ehrlich's haematoxylin	Potassium alum	Nuclear stain and also stains mucin, bone and cartilage	20–30 min	Natural ripening. Stain is more long-lasting
Harris haematoxylin	Ammonium or potassium alum	Good nuclear stain in exfoliative cytology	5–15 min in case of regressive stain and 5–30 s in progressive stain	Ripening by mercuric oxide
Gill's haematoxylin	Aluminium sulphate	Good nuclear stain	Regressive stain: 5–15 min	The stain is stable for 1 year
Cole's haematoxylin	Potassium alum	Good nuclear stain	Regressive stain: 30 min	Stable for 3 months

known as ripening. The oxidation process is slow and takes approximately 3 months. However, the useful staining life of the dye is longer by natural ripening process.

(b) *Chemical*: This is done by treating the dye with hydrogen peroxide or sodium iodate or mercuric oxide. Sodium iodate is the most commonly used oxidizing agent. The conversion of haematoxylin to haematein is instant; however the dye has short useful life span.

Dye-Mordant Complex Haematein is a weak anion and cannot combine with nucleic acid in the nucleus. When a metallic salt (mordant) is combined with haematein, then a cationic dye-metal complex is formed that behaves as a strong basic dye and combines with nucleic acid (Fig. 8.1). The type of mordant determines the type of tissue affinity of the dye and the colour of the stain. Commonly aluminium (Al^{3+}), iron (Fe^{3+}), molybdenum, tungsten and lead salts are used as mordant.

Types of Haematoxylin Haematoxylin can be classified according to its combination with different mordants such as:

1. Iron haematoxylin
2. Alum haematoxylin
3. Tungsten haematoxylin
4. Lead haematoxylin
5. Molybdenum haematoxylin
6. Only haematoxylin (no mordant attached)

Table 8.1 highlights different types of haematoxylin with their mordant and properties.

8.3 Bluing

The most of the regressive staining of haematoxylin needs bluing. The removal of excess hydrogen ion from the stain is known as bluing. Here the haemalum which is soluble is converted to insoluble form. Bluing gives crisp blue colour of the nuclei. In the process of bluing, the pH of the solution is raised to 8.5 (alkaline side). The tissue section is treated with alkaline reagent, and the acidic reagents are neutralized in bluing process. Bluing is done by the following methods:

- Running tap water for several minutes
- Treating the section by Scott's tap water (pH is 8): 2–3 min
- Ammonium hydroxide (5%): 2–3 min
- Ammonia vapour: few seconds

Scott's tap water:

Sodium bicarbonate: 2 g
Magnesium sulphate (anhydrous): 10 g
Water: 1 l

Slowly add magnesium sulphate in water so that it dissolves and heat is dissipated.

Warning The higher pH of the bluing agent makes the bluing more deeper blue colour quickly. However be careful the tissue section in high pH may be shed out from the slide.

8.3.1 Preparation of Different Haematoxylins and Their Properties

8.3.1.1 Harris Alum Haematoxylin

This haematoxylin is a regressive stain and is widely used in exfoliative cytology as a nuclear stain. Eosin is used as cytoplasmic stain. Ammonium or potassium alum is used here as mordant. This haematoxylin takes 5–15 min in case of regressive staining and 5–30 s in progressive staining.

Preparation of the stain:

Haematoxylin: 5 g
Absolute alcohol: 50 ml
Ammonium alum: 100 g
Distilled water: 1000 ml
Mercuric oxide: 2.5 g
Glacial acetic acid: 40 ml

Steps:

- Haematoxylin is dissolved in absolute alcohol.
- Alum in hot water is added.
- Heat to boil.
- Mix both the solution.
- Now slowly add mercuric oxide.
- Heat again till the colour changes to dark purple.
- Rapidly cool the flask by dipping in cold water.
- Add glacial acetic acid in the cold solution.

Cautions:

- Addition of glacial acetic acid is optional. It gives crisp staining but reduces the life span of the stain.
- The life span of the stain decreases within 2–3 months. The formation of precipitate and the increased stain time indicate that the stain material is deteriorating. In such situation, always filter the stain solution before use and also increase the time of staining.

8.3.2 Mayer's Haematoxylin

Mayer's haematoxylin is used predominantly as a nuclear counterstain where the cytoplasmic material is needed to demonstrate such as in enzyme histochemistry, PAS or mucicarmine stain. Potassium or ammonium alum is used as mordant. Mayer's haematoxylin is used in progressive staining, and it is best suited in automated staining procedure.

Preparation of the stain:

Haematoxylin: 1 g
Potassium or ammonium alum: 50 g
Sodium iodate: 0.2 g
Citric acid: 1 g
Chloral hydrate: 50 g
Distilled water: 1000 ml

Steps:

- Dissolve haematoxylin, potassium alum and sodium iodate in distilled water.
- Gently heat the solution and stir.
- Cool the solution in room temperature.
- Add chloral hydrate and citric acid in the solution.
- Boil the mixture for 4–5 min.
- Cool the solution.
- Filter.

The differences of Mayer's and Harris haematoxylin are highlighted in Table 8.2:

8.3.3 Ehrlich's Haematoxylin

It is a strong stain for the nuclei and also mucin, bone and cartilage. The stain is oxidized naturally by keeping it in air and sunlight for 2-month period. The bottle of stain can be kept in sunlight near window. Once the stain is matured, it can be used in laboratory.

Table 8.2 Differences between Mayer's and Harris haematoxylin

Factors	Harris haematoxylin	Mayer's haematoxylin
Solvent	Absolute alcohol	Distilled water
Oxidant	Mercuric oxide	Sodium iodate
Substance added to sharpen the stain	Glacial acetic acid	Chloral hydrate
Stain type	Both progressive and regressive stain	Progressive stain

Preparation of the stain:

Haematoxylin: 2 g
Absolute alcohol: 100 ml
Distilled water: 100 ml
Glycerine: 100 ml
Glacial acetic acid: 10 ml
Potassium alum: 10–15 g

Steps of preparation:

- Dissolve haematoxylin in absolute alcohol completely.
- Add the other ingredients.
- Finally add potassium alum (10–15 g) till the full saturation occurs.
- Keep the whole flask in sunlight for 2 months for maturation.

Note In case of any emergency situation to use the dye, one can add sodium iodate 100 mg in this solution for rapid maturation. In strict sense, then this haematoxylin solution should not be called as "Ehrlich's haematoxylin".

8.3.4 Cole's Haematoxylin

Cole's haematoxylin is an alum haematoxylin which is oxidized by iodine in alcohol. This is used as regressive nuclear stain.

Preparation of the stain:
Haematoxylin: 1.5 g
Saturated aqueous potassium alum: 700 ml
Iodine (1%) in absolute ethanol (95%): 50 ml
Distilled water: 250 ml
Steps of preparation:

- Add haematoxylin in distilled water.
- Gently heat and dissolve it completely.
- Mix iodine solution and alum.
- Boil.
- Cool and filter the solution.

8.4 Counterstain by Eosin

Eosin is used as counterstain of cytoplasm. It also stains the connective tissue and matrices. It stains rosy red to pink colour. This is a xanthene group of dye with a chemical structure "tetrabromofluorescein". Three types of eosin are seen: eosin Y, eosin B and ethyl eosin. Eosin Y is the most widely used eosin among these three types. The term Y indicates yellowish. Eosin is soluble in both water and alcohol.

Aqueous solution: 1% eosin in distilled water

Alcohol solution: 0.5% in 95% ethyl alcohol

Washing the eosin stained sections by tap water helps in the differentiation of the eosin staining.

8.5 Routine Haematoxylin and Eosin Stain

Requirements:

- Haematoxylin solution
- Grades of alcohol
- 1% HCl in 70% alcohol
- 1% eosin
- Xylene

Steps (*Fig. 8.2*):

- *Deparaffinization*: Keep the sections in xylene for 10 min each and three changes.
- *Rehydration*:
  - Absolute alcohol: 1–2 min
  - 95% alcohol: 1–2 min
  - 80% alcohol: 1–2 min
  - 60% alcohol: 1–2 min
- Rinse in tap water.
- *Nuclear stain by haematoxylin*: Harris haematoxylin 15 min (keep according to the type of haematoxylin).
- *Differentiation*: In case of regressive staining, one to two dips in acid alcohol (1% HCl in 70% alcohol) for differentiation are necessary. Please check by microscope for the desired staining of nucleus.
- Rinse in water.
- *Bluing*: Wash by running tap water for 10–15 min.
- *Counterstain by eosin*: 1% aqueous eosin Y for 2–3 min.
- *Dehydration*:
  - 95% alcohol: 3 min

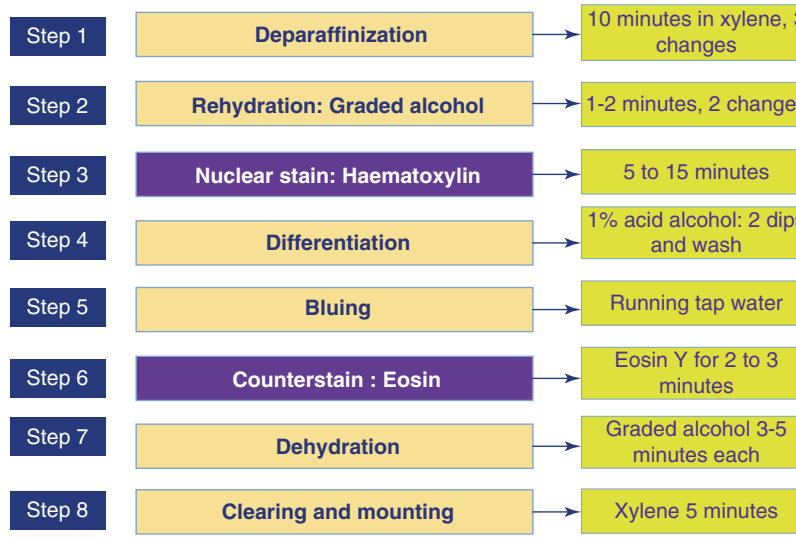

Fig. 8.2 Basic steps of haematoxylin and eosin stain are highlighted

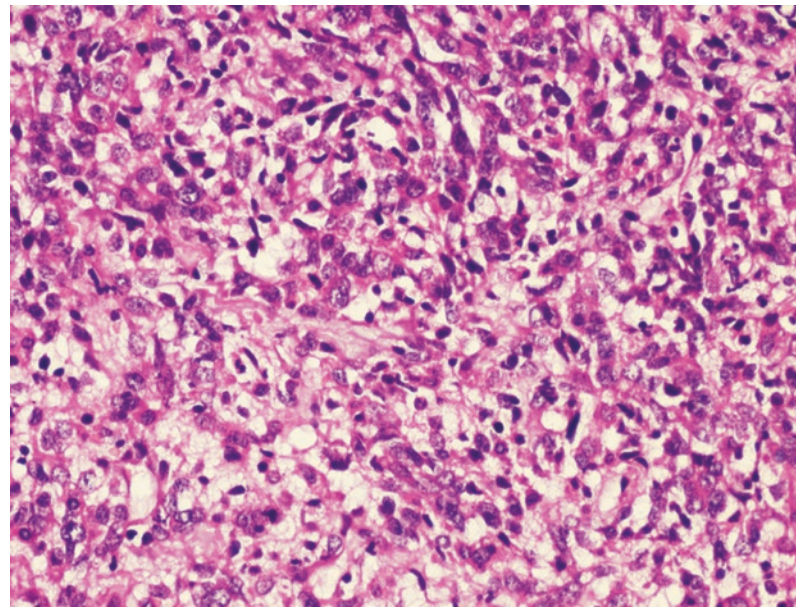

Fig. 8.3 Well-stained tissue by haematoxylin and eosin stain (haematoxylin and eosin stain X 200)

  - Absolute alcohol: 3 min
  - Absolute alcohol: 5 min
- *Clearing*: Xylene 5 min each in two jars.
- Mount in DPX.

Result (Fig. 8.3) Nuclei: blue to bluish black

Cytoplasm: pinkish

Troubleshooting in haematoxylin staining has been mentioned in Table 8.3 (Figs. 8.4, 8.5 and 8.6)

8.6 Iron Haematoxylin

In this type of haematoxylin, various iron salts are used as mordant such as ferric chloride or ferric ammonium sulphate. The different types of iron haematoxylin are:

1. Heidenhain's iron haematoxylin
2. Weigert's iron haematoxylin
3. Verhoeff's haematoxylin
4. Loyez haematoxylin

Table 8.3 Troubleshooting in haematoxylin staining

Problems	Possible causes	Remedies
Pale-stained nuclei	1. Too much differentiation 2. Too less time in haematoxylin 3. Due to excessive decalcification 4. Haematoxylin is over oxidized	1. Stain in haematoxylin again 2. Keep in haematoxylin for longer duration 3. Not possible to correct 4. Change the haematoxylin solution
Darkly stained nuclei	1. Too short differentiation 2. Too much time in haematoxylin 3. Thick section	1. Decolorize and do optimum differentiation 2. Decolorize and give appropriate time in haematoxylin 3. Recut thin section
Nuclei looks reddish brown	1. Insufficient bluing 2. Haematoxylin is degenerating	1. Restain by giving more time in bluing step 2. Check the oxidation status of haematoxylin
Pale-coloured cytoplasm by eosin	1. Too thin section 2. The eosin solution has pH more than 5 3. Too much dehydration of the section in alcohol	1. Recut the section properly 2. This may be due to dilution of eosin by the carryover bluing solution. Check pH of eosin solution, and if necessary, adjust pH by adding acetic acid 3. Do not keep the slide in alcohol for a long time
Cytoplasmic staining is very dark	1. Long duration in eosin solution 2. Overconcentrated eosin solution 3. Very quick dehydration in alcohol	1. Keep the section in eosin for shorter duration 2. Make optimally diluted eosin solution 3. Increase time duration in dehydration
Bluish-black precipitate	It may be due to precipitation of haematoxylin	Filter the haematoxylin staining solution
Staining is irregular and spotty	Improper deparaffinization	Keep the slide in xylene for longer time to remove the paraffin
Dark-blue stain at the edge of the tissue sections	Due to heating artefact for using electrocautery	No solution
Water bubbles in the sections	Incomplete dehydration	Remove the mounting medium and coverslip. Keep the section in absolute alcohol for dehydration. Do several changes and then remount
Milky section after the xylene rinse before putting the coverslip	Incomplete dehydration	Change the alcohol solution. Please dehydrate the section properly before putting in xylene

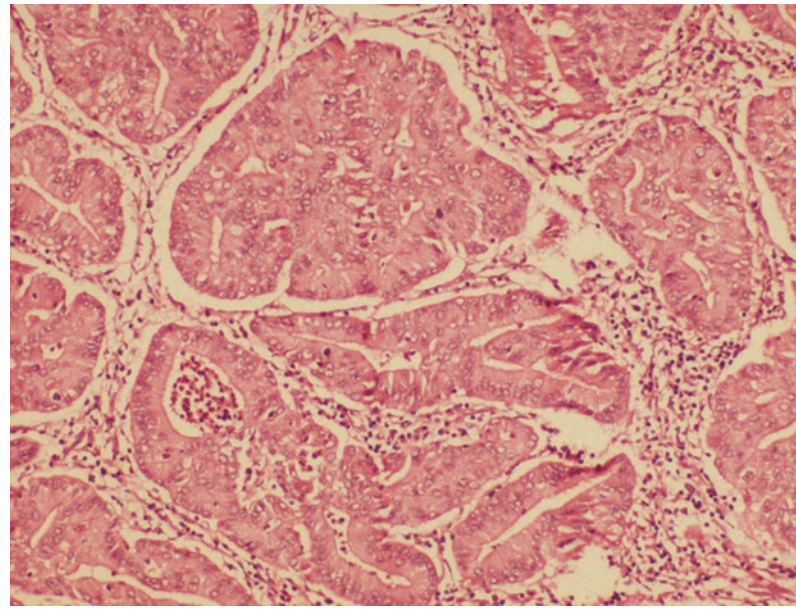

Fig. 8.4 Pale-stained nuclei (haematoxylin and eosin stain X 200)

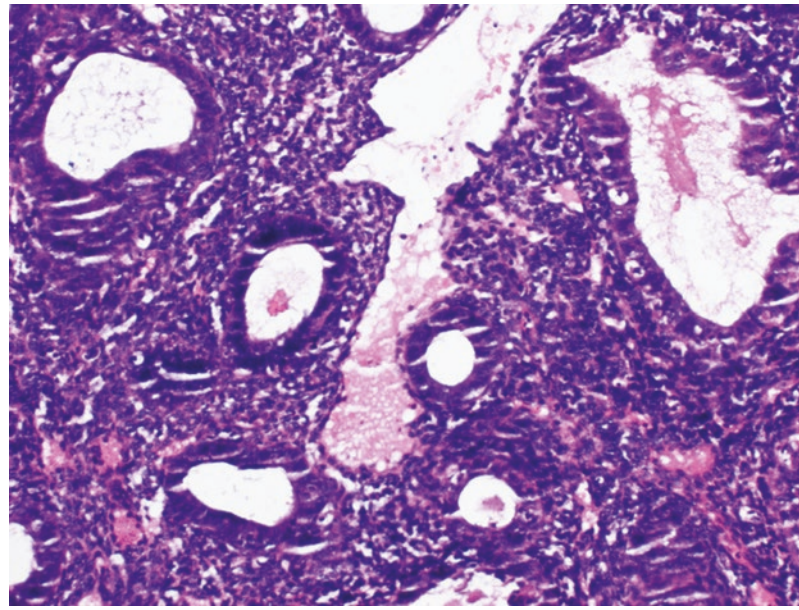

Fig. 8.5 Too dark nuclear stain (haematoxylin and eosin stain X 200)

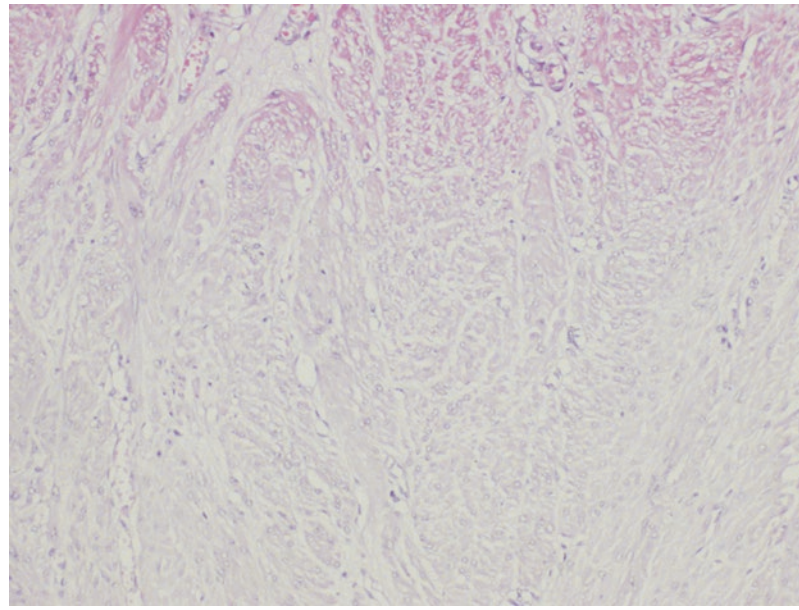

Fig. 8.6 Imperfect deparaffinization of the tissue section (haematoxylin and eosin stain X 200)

8.6.1 Heidenhain's Iron Haematoxylin

Heidenhain's iron haematoxylin stains the tissue jet black. It stains various cellular constituents such as mitochondria, chromatin and nucleoli. In addition it also stains myelin sheath and striations of the muscle. This gives regressive stain. Iron alum is used as an oxidizing agent of haematoxylin, and ferric ammonium sulphate is used as mordant. The approximate time of staining is 30 min.

8.6.1.1 Preparation

Solution 1

Haematoxylin: 0.5 g
Absolute alcohol: 10 ml
Distilled water: 90 ml

Steps:

- Haematoxylin is dissolved completely in absolute alcohol.
- Add distilled water.
- Keep the solution for 4–6 weeks for ripening.

Solution 2

Ferric ammonium sulphate: 5 g
Distilled water: 100 ml
Step: Dissolve violet crystal of ferric ammonium sulphate in distilled water.
Staining

- Dewax the tissue.
- Absolute alcohol.
- 95% ethyl alcohol.
- Keep section in mordant solution (solution 2) for 60 min.
- Rinse in distilled water.
- Stain by solution 1 (Heidenhain's haematoxylin 0.5%) for 60 min.
- Rinse in water.
- Differentiate in 5% alum solution.
- Wash in running water: 5–10 min.
- Dehydrate.
- Clean by xylene.
- Mount in DPX.

Result Target tissue takes black stain.

8.6.2 Verhoeff's Iron Haematoxylin

Verhoeff's iron haematoxylin is used to stain elastic tissue. The stain provides very good contrast for microphotography. Ferric chloride is used here as mordant. Strong iodine is used for oxidizing agent. The whole solution should be prepared just before use because the solution may be over oxidized if kept more than 1 h.

8.6.2.1 Preparation

No. 1:
Haematoxylin: 5 g
Alcohol (100%): 100 ml
No. 2:
Ferric chloride: 10 g
Distilled water: 100 ml
No. 3:
Iodine: 1 g
Potassium iodide: 2 g
Distilled water: 100 ml
No. 4: Working solution
Add:
No. 1: 40 ml
No. 2: 16 ml
No. 3: 16 ml
Mix those solutions in order**.**
Steps:

- Dewax.
- Serial grade of alcohol for hydration.
- Stain by freshly prepared working haematoxylin solution for 10 min.
- Rinse in water.
- Differentiation: by 2% ferric chloride.
- Wash by tap water.
- Remove iodine by 95% ethyl alcohol: 5 min.
- Counterstain: 1% eosin for 1–2 min.
- Dehydrate.
- Clean by xylene.
- Mount by DPX.

Result Elastic fibre takes black stain.

8.6.3 Tungsten Haematoxylin

Tungsten haematoxylin is used to demonstrate nerve tissue (such as astrocyte), muscle tissue and collagen. Here phosphotungstic acid is used as mordant.

8.6.3.1 Preparation

Solution A
Haematein: 0.8 g
Distilled water: 1 ml
Step: Dissolve 0.8 g haematein in 1 ml distilled water.
Solution B
Phosphotungstic acid: 0.9 g
Distilled water: 9 ml

Step: Mix both solutions A and B to get the final staining solution.

Staining

- Dewax.
- Serial grade of alcohol for hydration.
- Treat by 0.25% potassium permanganate for oxidation—5 min.
- Wash in distilled water.
- Bleach: by oxalic acid (5%) for 5 min.
- Wash in tap water.
- Stain: PTAH final solution for 12–24 h in room temperature.
- Wash in distilled water.
- Quick dehydration by 95% alcohol and absolute alcohol.
- Clean by xylene.
- Mount by DPX.

Note: Quick dehydration prevents the removal of red stain by alcohol.

Result Connective tissue materials such as collagen, reticulin fibres, etc.: red

Nuclei, centrioles, striated muscle, etc.: blue

The different uses of haematoxylin are highlighted in Table 8.4.

Table 8.4 Applications of different haematoxylin stains for different purposes

Substances	Haematoxylin
Routine stain in histology sections and cytology smears	Harris haematoxylin
Carbohydrates	Mayer's haematoxylin
Phospholipid	Baker's acid haematein technique
Fibrin, cross striations of skeletal muscle	Tungsten haematoxylin
Connective tissue fibres	Verhoeff's iron haematoxylin
Amoeba, microfilaria	Iron haematoxylin
Nuclear chromatin	Gill's haematoxylin
Photomicrography	Heidenhain's iron haematoxylin
Counterstaining in immunohistochemistry and cytochemistry	Ehrlich's haematoxylin

8.7 Clearing of the Smear

Removal of alcohol or clearing of the sample is done by putting the sample in xylene. The chemical compound of xylene is dimethyl benzene. The clearing agent should be colourless and its refractive index should be close to the mounting media and coverslip (Box 8.1).

8.7.1 Mounting

The main function of the mounting media is to give a protective cover over the smear and to make a permanent bond between the coverslip and the slides (Box 8.2). Mounting medium should have following properties:

- The same refractive index of the coverslip and glass slide. The refractive index of the mounting medium should be 1.52–1.54.
- Mounting media should be colourless.
- It should quickly dry and stick to the slide.
- It should resist contamination particularly the growth of microbes.
- It should not react with the stain or tissue.
- It should be miscible with clearing agent.
- A neutral pH to prevent fading of the stain.

- Low viscosity; otherwise there may be air bubble formation at the time of putting the coverslip. This air bubbles are brownish in colour and are known as a *cornflake artefact.*

Box 8.1 Clearing Agent

- Xylene (dimethyl benzene)

Basic properties:

- Colourless.
- Refractive index should be the same as the mounting media and coverslip.
- It gives transparent cytoplasm.

Warning: toxic

Box 8.2 Mounting medium
Aim: to give a protective cover over the smear and to make a permanent bond between the coverslip and the slides

Ideal mounting medium:

- The same refractive index of the coverslip and glass slide (1.52–1.54).
- Colourless.
- It should quickly dry and stick to the slide.
- Resist the growth of microbes.
- No reaction with the stain or tissue.
- Miscible with clearing agent.
- A neutral pH to prevent fading of the stain.
- Low viscosity.

Types of mounting medium:

- Neutral resins: such as Canada balsam and Euparal
- Synthetic resins: DPX
- Aqueous media: glycerine, polyvinyl alcohol, etc.

Permanent mounting: DPX
Temporary mounting: glycerine-glycerol, polyvinyl alcohol

Types of Mounting Media There are three types of mounting media:

- Neutral resins: such as Canada balsam and Euparal
- Synthetic resins: DPX
- Aqueous media: glycerine, polyvinyl alcohol, etc.

Neutral Resins The commonly used neutral resin is Canada balsam. The refractive index of it is 1.523. Canada balsam is well soluble in xylene and is made as:

Canada balsam: 60 g
Xylene: 100 ml

Disadvantages:

- Yellow staining after some time.
- Takes time to dry.
- The basic dyes are poorly preserved.

Canada balsam is not used nowadays.

Synthetic Resins DPX is the most widely used synthetic resin with a refractive index 1.523. It is called DPX as it contains:

D = distyrene
P = plasticizer (tricresyl phosphate)
X = xylene

DPX is colourless and preserves the stains very well. It dries also very quickly.

DPX should be used liberally over the slide, and the excess DPX should be wiped off from the coverslip margin.

Disadvantage:

- It frequently retracts from the margins of the coverslip. This can be prevented by adding a plasticizer that makes a mesh with plastic [3].

Aqueous Media The aqueous media are used when the stains are affected by dehydration by alcohol or clearing by xylene, e.g. fat stain by Sudan black, needs aqueous media.

Glycerine-glycerol: This is the commonly used aqueous media for temporary mounting.

Polyvinyl alcohol: This is alternative to glycerine jelly and is often used in immunofluorescence or frozen section of lipid stains.

Table 8.5 highlights the comparison of different mounting media.

Application of mounting medium:

- Put one to two drops of mounting medium on the middle of the tissue section over the slide.
- Select an appropriate coverslip for the section.
- Rapidly invert the slide over the coverslip.
- Mounting medium slowly spreads under the coverslip.

Table 8.5 The comparison of different mounting media

Mounting medium	Refractive index	Common use	Advantages	Disadvantages
Canada balsam	1.523	Permanent mounting	Well soluble in xylene	– Yellow staining after some time – Takes time to dry – The basic dyes are poorly preserved
DPX	1.523	Permanent mounting	– Preserve the standard stains – Quickly dry	– Retraction of margin of coverslip
Glycerine-glycerol	1.47	– Temporary mounting – Oil red O and Sudan black stain – Fluorescent stain	– Inexpensive – Safe – Quick to apply	– Unsuitable for prolonged preservation, and the coverslip margin should be sealed
Polyvinyl alcohol	1.5	– Temporary mounting – Fat stain – Fluorescent stain	Safe and good alternative to glycerine-glycerol	– Unsuitable for prolonged preservation

Cautions:

- Too small amount of mounting medium: Air bubbles may appear.
- Too much amount of mounting medium: It may spread beyond the edges of coverslip, and the sample may also float.

8.7.2 Coverslip

Coverslip gives a protective covering over the smear and prevents fading of the stain or any further physical damage. The good coverslip should have following characteristics:

- Clear glass of 0.130–0.170 mm thickness
- Plane surface and straight margin
- Sufficiently wide to cover the smear

Liquid coverslip is equally effective. Nowadays many laboratories use automatic coverslip machine.

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9 Special Stains for the Carbohydrate, Protein, Lipid, Nucleic Acid and Pigments

9.1 Introduction

Other than routine haematoxylin and eosin stain, various special stains are now essential parts in routine laboratory works. Box 9.1 highlights the overall indications of these special stains in laboratory. In this chapter we will discuss the basic principles, applications and techniques of different stains.

Table 9.1 shows the list of commonly used special stains in histopathology and cytology laboratory.

Box 9.1 Applications of Special Stain

- Demonstration of various cellular products for diagnosis
  - Carbohydrates
  - Proteins
  - Lipids
  - Pigments
- Demonstration of extracellular material for the identification of diseases such as amyloid
- Identification of microbial organisms
- Estimation of DNA and RNA content of the cell

Table 9.1 Commonly used stain for different substances

Material	Stain
Carbohydrate	• Periodic acid-Schiff (PAS) • Alcian blue • PAS and Alcian blue • Mucicarmine
Lipid	• Oil red O • Sudan black • Ferric haematoxylin
Nucleic acid	• Feulgen stain • Acridine orange • Methyl green-pyronin
Hemosiderin pigment	• Perls' reaction
Bile pigment	• Fouchet's stain
Melanin	• Masson-Fontana method • Schmorl's stain

9.2 Carbohydrates

Carbohydrates are the compounds that contain polyhydroxyaldehyde or polyhydroxyketone groups. They are represented by the common formula $C_n(H_2O)_m$. The carbohydrates can be classified depending on the number of subunits as monosaccharide, oligosaccharide and polysaccharide. They are also further classified depending on their binding with protein and lipid material.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_9

Box 9.2 Classification of Carbohydrate

A. Simple carbohydrates
   - Monosaccharide: e.g. glucose, galactose
   - Oligosaccharide: e.g. maltose, sucrose
   - Polysaccharide: e.g. glycogen, starch

B. Glycoconjugates (complex carbohydrates)
   - Acid mucopolysaccharides
     - Carboxylated: e.g. hyaluronic acid
     - Sulphated: e.g. chondroitin sulphate
   - Mucin
     - Neutral mucin: Surface epithelial cells of the stomach
     - Acidic mucin
       Sialomucin: Goblet cells, salivary glands
       Sulphomucin: Mucous glands of the bronchus
   - Others
     - Glycolipid
     - Membrane protein
     - Blood group antigen

9.2.1 Simple Carbohydrates

Monosaccharides These are the simplest form of carbohydrates with the empirical formula $(CH_2O)_n$. They are the building blocks of various other carbohydrates. They contain aldehyde or ketone group and varying number of carbon atoms (5C atoms = pentose, 6C atoms = hexose, etc.). The monosaccharides are water soluble and therefore difficult to demonstrate in routine histology section.

Example: Glucose, ribose, fructose, etc. Figure 9.1 shows the structure of glucose molecule.

Oligosaccharides (Fig. 9.1) These are the polymers of monosaccharides that contain 2–10 monosaccharide units.

Example: Sucrose, lactose, maltose

Polysaccharide (Fig. 9.1) Polysaccharide consists of multiple monosaccharides that are linked by covalent bonds.

Example: Glycogen, starch. Glycogen is available in large quantity in the liver followed by the skeletal and cardiac muscles.

Glycoconjugates or Proteoglycans These are primarily groups of proteins that are extensively glycosylated. The proteoglycans have a central core protein that is covalently linked with polysaccharide. The carbohydrate part of the proteoglycan is known as glycosaminoglycans. The different types of glycosaminoglycans include:

- Chondroitin sulphate: Present in the cartilage, ligament, bone
- Dermatan sulphate: Present in the skin
- Heparan sulphate: Present in the aorta
- Heparin: Present in granules of mast cell
- Hyaluronic acid: Present in synovial fluid

Mucin Mucins are glycoproteins of high molecular weight and are composed of a polysaccharide chain and a protein component.

Mucin = 80% carbohydrate (hexosamine-containing carbohydrate) + 20% protein

Mucin covers the epithelial cells and makes a physical barrier that protects the cells from any external injury [1]. Mucin may be of two types: (1) secretory mucin, secreted in the respiratory tree, gastrointestinal tract and cervical part of female genital tract, and (2) membrane-associated mucin, the mucin attached with the membrane of cells. Mucin may also be noted in non-epithelial tissues.

The amino acid components of the protein core may be variable, and depending on the tandem repeats of the nucleotide sequence of the amino acid of the protein component of mucin, it may be classified as many distinct functional types of mucin [2, 3] (Table 9.2). The MUC genes are tissue specific and have distinct biophysical and biochemical properties. So far 20 such MUC genes have been described.

The carbohydrate part of mucin consists of 80% of the molecular weight of mucin. The polysaccharide part of the mucin may be neutral, weakly acidic or strongly acidic.

Neutral mucin: The name itself indicates that the polysaccharide chain here is neutral. Neutral

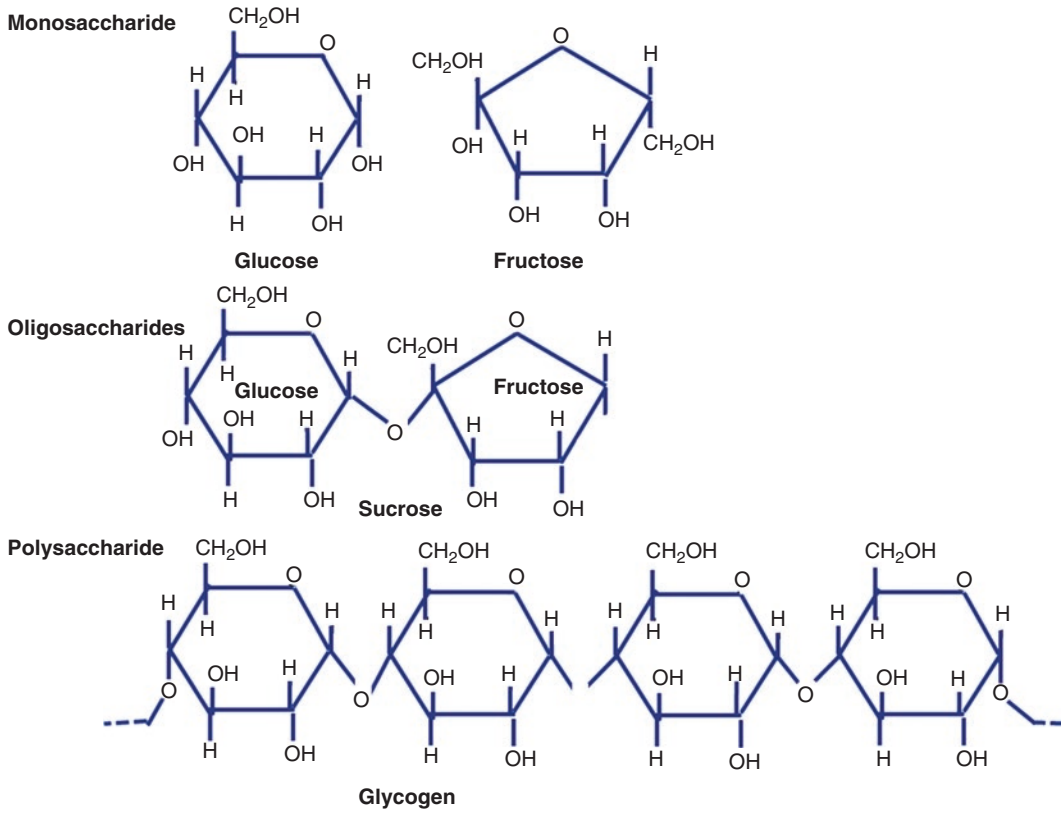

Fig. 9.1 Structure of monosaccharide, oligosaccharide and polysaccharide

Table 9.2 Tissue specificity of MUC genes

MUC gene	Normal locations	Tumour and MUC
MUC 1	Bronchi, breast, urinary bladder, kidney, stomach, pancreas, gall bladder, prostate, cervix	Majority of adenocarcinomas are positive for MUC 1. Adrenocortical and hepatocellular carcinomas are always negative for MUC 1
MUC 2	Small and large intestine, salivary gland and endometrium	Colonic carcinomas are positive for MUC 2
MUC 3	Salivary gland, gall bladder, small and large intestine	Colonic carcinomas, pancreatic carcinoma
MUC 4	Bronchus, endocervix, stomach, small and large intestine	Increased in pancreatic, breast, lung and ovarian carcinomas
MUC 5AC	Bronchus, stomach, endocervix	Endocervical adenocarcinoma and gastrointestinal carcinomas are positive
MUC 6	Endocervix, endometrium Stomach, gall bladder, ileum	Gastric carcinomas show highly expressed MUC 6. In colonic carcinoma MUC 6 is increasingly expressed along with low MUC 2
MUC 7	Sublingual and submandibular glands	Markers of urothelial carcinoma, and the demonstration of MUC 7 in the lymph node identifies micrometastasis from the bladder

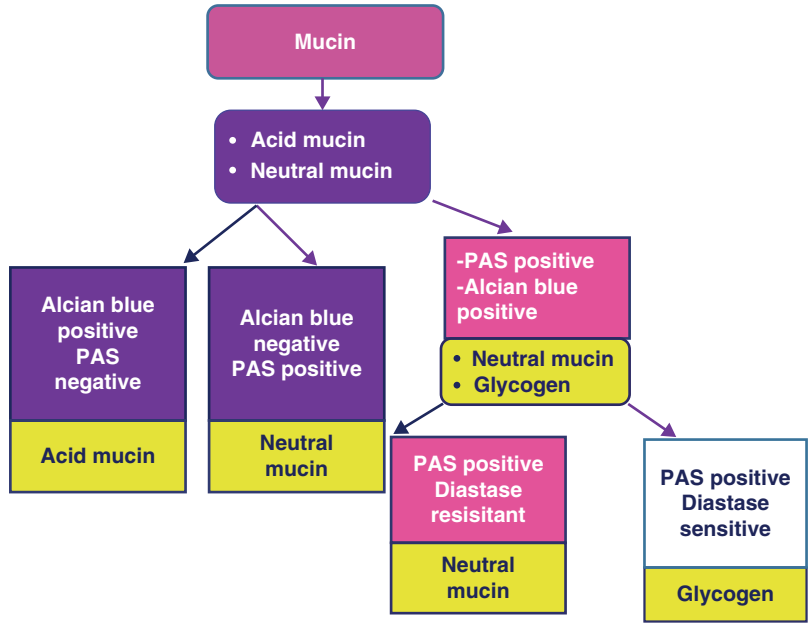

Fig. 9.2 Flow diagram shows different stains for mucin

mucin is noted in surface epithelial cells of the stomach and prostate and in Brunner's gland of the duodenum. These mucins are positive for periodic acid-Schiff's stain and negative for Alcian blue stain.

Acidic mucin: Here the polysaccharide chain is anionic. These mucins are positive for Alcian blue stain in low pH (2.8).

Sialomucin: It contains sialic acid. Sialic acid is derived from the acetylation of neuraminic acid. This is a weak acidic mucin. Sialomucin is found in goblet cells, salivary glands, etc.

Sulphomucin: Sulphomucin contains the sulphate group and it is a stronger acidic mucin. This type of mucin is seen in the mucous glands of the bronchus.

Strongly sulphated acid mucins: This type of mucin consists of

- Connective tissue mucin: chondroitin sulphate, keratan sulphate, heparin sulphate
- Bronchial glands
- Some fractions of goblet cells of the intestine

Strongly sulphated mucins are PAS negative and Alcian blue positive at pH 0.5.

Weakly sulphated acid mucins: They are epithelial mucins. This group consists of:

- Colonic goblet cells
- Bronchial glands

Weakly sulphated acid mucins are Alcian blue positive at pH 1 and negative for PAS.

Both neutral and acidic mucins are positive for mucicarmine.

Figure 9.2 highlights the staining pattern of different types of mucin.

9.3 Staining of Different Carbohydrates

9.3.1 Glycogen

Glycogen, the polysaccharide, is demonstrated by periodic acid-Schiff's (PAS) reaction.

9.3.1.1 Periodic Acid-Schiff's (PAS) Stain [4]

PAS stain demonstrates neutral polysaccharides that are present in the basement membrane and also secretion of various glands in our body.

Indications to do PAS stain

- *To demonstrate polysaccharides*: PAS helps to demonstrate glycogen, cellulose and starch. It

demonstrates glycogen in glycogen storage disorders. Basement membrane of the glands, glomeruli, etc. can also be demonstrated by PAS stain. The capsule of the various fungi such as cryptococci, histoplasma, blastomycosis, etc. containing carbohydrate material is demonstrated by PAS,

- *Glycoprotein*: Mucin, particularly neutral mucin, is demonstrated by PAS. The stain is helpful to stain mucin of endocervical glands, intestinal glands and bronchial glands.
- *Glycolipid*: PAS helps to demonstrate cerebrosides and gangliosides. Glucocerebrosides and galactocerebrosides are accumulated in Gaucher's and Krabbe diseases, respectively. Gangliosides are accumulated in rare lysosomal storage disease.
- *Pigments*: Certain pigments such as lipofuscin and pigments of Dubin-Johnson syndrome are demonstrated by PAS stain.
- *Plasma cells*: Russell bodies of plasma cells are stained by PAS.

Principle (Fig. 9.3)

- The hydroxyl group (OH) of the carbohydrate molecule is oxidized to aldehyde (CHO) group by periodic acid.
- These aldehyde groups react with Schiff's reagent to form a magenta-coloured compound.

Components of Solutions

Solution 1: Periodic acid (1%)	
Periodic acid	1 g
Distilled water	100 ml
Solution 2: Schiff's reagent	
Basic fuchsin	1 g
Distilled water	200 ml
Potassium metabisulphite	2 g
1 N hydrochloric acid (HCl)	20 ml
Activated charcoal	2 g

Preparation

- Dissolve basic fuchsin (1 g) in 200 ml of boiling distilled water.
- Cool the solution.
- Add 1 N hydrochloric acid and mix well.

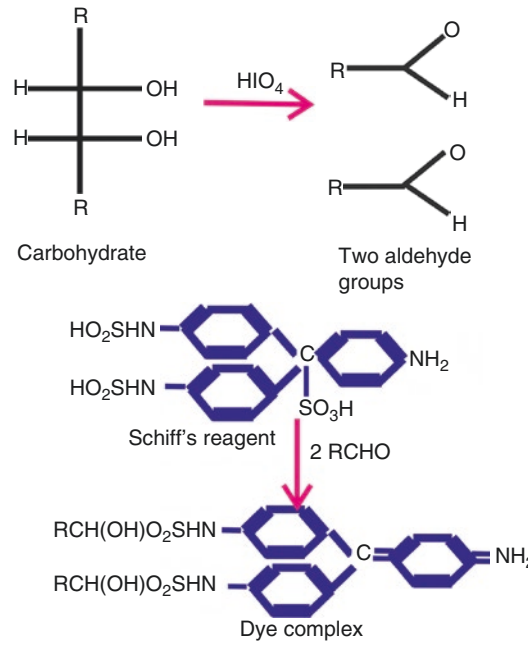

Fig. 9.3 Schematic diagram shows basic principle of PAS stain. Periodic acid converts the hydroxyl (OH) group to aldehyde group (CHO) that reacts with Schiff's reagent to give a magenta colour

- Add potassium metabisulphite (2 g).
- Add activated charcoal (2 g).
- Keep the solution in the dark.

Steps

1. Deparaffinize.
2. Pass through graded lower concentration of alcohol and section/smear to bring in water.
3. Oxidize with periodic acid (1%) for 5–10 min.
4. Clean with water.
5. Keep in Schiff's reagent for 20–30 min.
6. Clean in running tap water for 5 min.
7. Counterstain with haematoxylin.
8. Wash in tap water for blueing.
9. Dehydrate in absolute alcohol.
10. Clear in xylene.
11. Mount.

Result

Glycogen and glycoprotein: Magenta colour

Materials Positive for PAS Reaction Glycogen, starch, mucin, reticulin, basement membrane, capsule of fungi, etc.

Testing Schiff's reagent

Add drops of Schiff's reagent to formalin. Active Schiff's reagent will quickly change the colour of formalin to pink.

9.3.1.2 Alcian Blue

Alcian blue stains *acid mucin* (in acidic pH 2.5), such as sialomucin and sulphomucin. It stains mucin of the salivary glands, prostate and large intestine. Alcian blue also stains proteoglycans of cartilaginous material.

Indications

- Intestinal metaplastic cells in Barrett's oesophagus and also in stomach biopsy: The intestinal metaplastic cells contain acid mucin, and these cells in Barrett's oesophagus are better demonstrated by Alcian blue.
- Mucinous adenocarcinoma of the ovary.
- Pleural mesothelial cell: The pleural mesothelial cells contain hyaluronic acid that are Alcian blue positive and sensitive to hyaluronidase enzyme. In comparison, adenocarcinoma cells are Alcian blue positive and resistant to hyaluronidase enzyme.
- Myxoma: Mucin-secreting tumours such as myxomas are positive for Alcian blue stain.
- Others: Mucinous materials in myxoedema, discoid lupus erythematous lesion, etc. are also demonstrated by Alcian blue stain.

Basic Principle Alcian blue is a group of water-soluble polyvalent basic dye. The dye is made of a copper-containing phthalocyanine ring with a copper atom in its centre. Th phthalocyanine ring is also attached with four isothiouronium groups that are positively charged (Fig. 9.4). This positively charged Alcian blue dye complex has an attraction with anionic sites of the mucin. Copper imparts the blue colour of the dye-mucin complex.

Solution

Alcian blue solution	
Alcian blue, 8GX 1% aqueous solution	1 g
Acetic acid (3%) solution	100 ml
Neutral red solution	
Neutral fast red	0.1 g
Aluminium sulphate	5 g
Deionized water	100 ml

Steps to make the solution

- Dissolve aluminium sulphate in deionized water and heat.

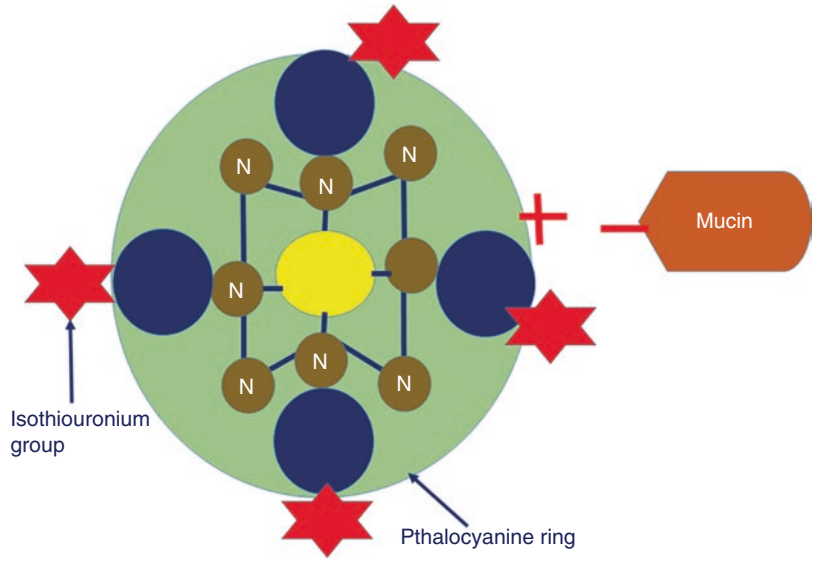

Fig. 9.4 Schematic diagram shows basic principle of Alcian blue stain. Copper-containing phthalocyanine ring is attached with four positively charged isothiouronium groups that have an attraction with anionic sites of the mucin. Copper imparts the blue colour of the dye-mucin complex

- Mix neutral fast red in hot water.
- Filter.

Method of staining

1. Deparaffinize.
2. Rehydration of the section/smear by graded alcohol.
3. Rinse in deionized water.
4. Keep the smear in Alcian blue for 30 min.
5. Rinse in running water: 5 min.
6. Counterstain with neutral fast red: 10 min.
7. Rinse in 95% ethyl alcohol.
8. Dehydrate in absolute alcohol.
9. Clean in xylene.
10. Mount.

Result Acid mucin (sialomucin, sulphomucin), proteoglycans, hyaluronic acid will take a blue colour.

9.3.2 Combined PAS-Alcian Blue Staining

Indications Combined use of Alcian blue and PAS in a same section helps to demonstrate both acidic and neutral mucin in the same section (Fig. 9.5). This is frequently applied in gastrointestinal biopsy sections.

Solution: Alcian blue, periodic acid and Schiff's reagent solution can be made as described before.

Method of staining

1. Deparaffinize.
2. Rehydration of the section/smear by graded alcohol.
3. Rinse in deionized water.
4. Keep the smear in *Alcian blue* for 30 min.
5. Wash in tap water followed by deionized water.
6. Oxidize with *periodic acid* (1%) for 5–10 min.
7. Clean in running tap water for 5 min.
8. Keep in *Schiff's reagent* for 20–30 min.
9. Clean in running tap water for 5 min.
10. Counterstain with *haematoxylin.*
11. Wash in tap water for blueing.
12. Dehydrate in absolute alcohol.
13. Clear in xylene.
14. Mount.

Result
Glycogen: Magenta colour
Acid mucin: Blue colour

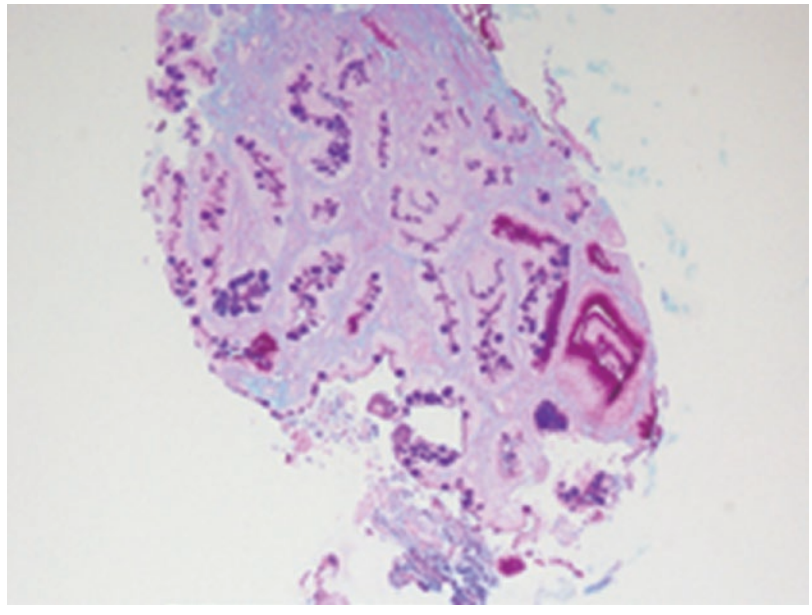

Fig. 9.5 The combined PAS and Alcian blue stain: PAS-Alcian blue stain at pH 2.5 highlighting the intestinal metaplasia (stained blue by Alcian blue) in an antral biopsy. The magenta stained mucin is the normal neutral mucin of the antrum (40×)

9.3.2.1 Mucicarmine Stain [5]

Indications

- Mucicarmine stain demonstrates acid mucin.
- It stains mucin of intestinal adenocarcinoma.
- The capsule of fungi such as cryptococci is stained by mucicarmine.

Principles The active dye molecule in mucicarmine stain is carmine. Carmine is composed of a multi-ring molecule carminic acid bound with aluminium ion. The carmine complex is positively charged, and so it binds with negatively charged anionic acid mucin (Fig. 9.6).

Applications Mucicarmine stain is applied to demonstrate acid mucin in the malignant cells of adenocarcinoma. It also stains capsule of *Cryptococcus*.

Solution

Southgate mucicarmine stock solution	
Carmine	1 g
Aluminium hydroxide	1 g
Alcohol (50%)	100 ml
Anhydrous aluminium chloride	0.5 g

Preparation

- Add carmine and aluminium hydroxide in 50% alcohol.
- Mix anhydrous aluminium chloride 0.5 g.
- Gently shake.

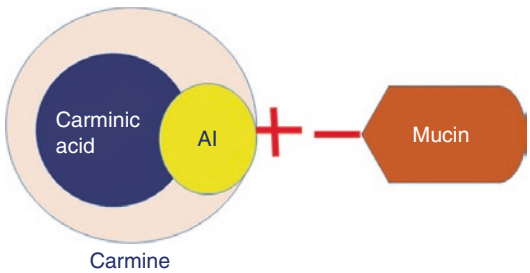

Fig. 9.6 Schematic diagram shows the basic principle of mucicarmine stain. The positively charged carmine complex binds with the negatively charged anionic acid mucin

- Boil the whole solution by keeping the flask in hot water bath.
- Cool.
- Filter the solution and preserve it in 4°C. This will be fit for use for 4–6 months.

Mucicarmine working solution

Mucicarmine stock solution	10 ml
Deionized water	90 ml

Steps

1. Deparaffinize.
2. Rehydration of the section/smear by graded alcohol.
3. Rinse in water.
4. Counterstain with haematoxylin for 5–10 min.
5. Wash in water.
6. Keep in mucicarmine solution for 30 min.
7. Rinse in water.
8. Rinse in 95% ethyl alcohol.
9. Dehydrate in absolute alcohol.
10. Clean in xylene.
11. Mount.

Interpretation Positive mucicarmine stain shows a dark red colour.

9.4 Lipids

The word lipid originated from the Greek word "lipos" which means fat. The lipids are defined as group of naturally available organic fatty substances that are soluble in alcohol and insoluble in water.

Lipids are the major components of the cell membrane and the membranous component of many cellular organelles. The myelin component of the nerve sheath is also made of lipid.

Lipids are classified as simple lipids, compound lipids and derived lipids (Table 9.3).

Simple Lipids These are the lipids which on hydrolysis produces fatty acid and glycerol (Fig. 9.7). *Example*: fats, oils and waxes

Table 9.3 Classification of lipids

Simple lipid	Compound lipids	Derived lipids
• Fatty acid • Simple triglycerides • Mixed triglycerides • Waxes	• Phospholipid – Glycerol based Phosphatidylcholine Phosphatidylserine Plasminogen – Sphingosine based Sphingomyelin – Phosphosphingosides – Phosphoinositides • Glycolipid – Cerebroside – Sulphatide – Ganglioside	• Steroids • Terpenes • Carotenoids

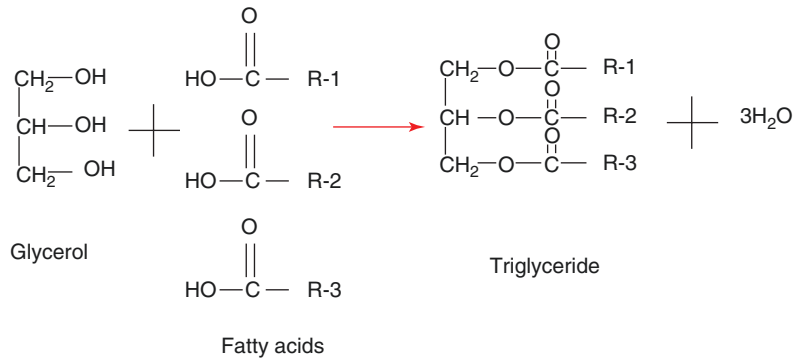

Fig. 9.7 The diagram shows the reaction of fatty acid and glycerol-forming triglyceride

Fats: Esters of fatty acid and glycerol

Wax: Esters of fatty acid and alcohol (other than glycerol)

Compound Lipids These are composed of fatty acid, alcohol and another group such as sulphur-, phosphorous- or carbohydrate-producing sulpholipid, phospholipid, glycolipid, etc.

Derived Lipids The derived lipids are the substances that are derived from the hydrolysis of simple or compound lipids.

Example: Fatty acids, steroids, cholesterol, carotenoids, etc.

Fixation

Most of the dyes are soluble in lipid more than their solvent. Therefore it is preferable to use frozen section tissue for lipid staining. The best fixative for lipid is formal calcium (10% formalin with 2% calcium acetate).

9.5 Stains

Figure 9.8 highlights various stains for lipids.

9.5.1 Oil Red O [6]

Indications Oil red O stain is used for demonstration of lipid material. The lipid material takes a deep red colour in this stain.

Principle Oil red O is a diazo dye that is highly soluble in lipid substance. Oil red O is dissolved in alcohol solution, and in fat-containing tissue, the dye leaves the solution and moves to the tissue fat as it is more dissolved in fat. As fat is dissolved in alcoholic solution so the processed tissue cannot be stained by oil red O and frozen section or fresh tissue is needed for this stain. The mounting medium of oil red O also should not be alcohol based. Aqueous mounting is used in oil red O stain.

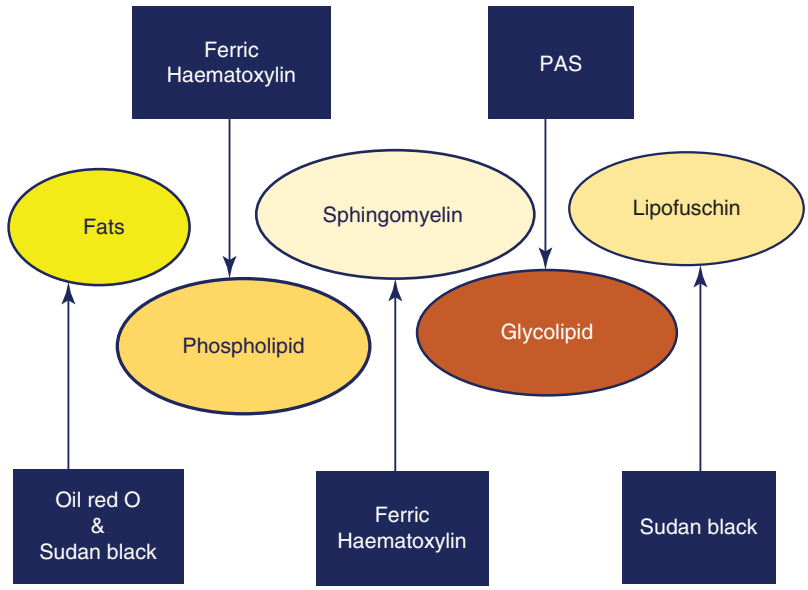

Fig. 9.8 Various stains for lipid materials in tissue section

Application Oil red O is particularly useful to demonstrate lipid in renal cell carcinoma. The lipoblasts are also oil red O positive.

Preparation of oil red o stain

Stock solution	
Oil red O	0.5 g
Isopropanol	100 ml
Dissolve oil red O in the alcohol and keep it overnight	
Working solution	
Oil red O stock solution	30 ml
Distilled water	20 ml

After mixing the stock solution with distilled water, wait for 10 min and then filter to use. The stability of the working solution is only 1 h.

Fixation Fresh frozen section or air-dried smear

Steps

1. Put the slides directly into oil red O solution for 20–30 min.
2. Rinse with running water.
3. Counterstain with haematoxylin for 30 s.
4. Rinse in water.
5. Mount in glycerine.

Result Oil red O stains lipid, lipoprotein and triglycerides. Lipid takes a red colour.

9.5.2 Sudan Black B [7]

Principle Sudan black B is a lipophilic dye and is insoluble in water. This dye therefore is dissolved in tissue fat and stains them. The slightly basic dye Sudan black B combines with the acidic component of the lipid.

Fixation Fresh frozen section or air-dried smear

Solution

Sudan black B	1 g
Propylene glycol	100 ml

Heat the solution gently up to 100 °C and then cool it and filter the solution.

Steps

- Fresh frozen section.
- Fix in 10% formalin if fresh section.
- Wash well in distilled water.
- Air-dry.
- Put in Sudan black B solution for 1–2 h.
- Rinse in ethyl alcohol (70%): twice for 2 min.
- Rinse in distilled water.
- Counterstain with haematoxylin.
- Rinse in water.
- Mount in glycerine jelly (aqueous media).

Result Fat takes dark black stain

9.5.3 Ferric Haematoxylin for Phospholipid [8]

Fixation Frozen section tissue

Preparation of solution

Solution 1	
Ferric chloride	2.5 g
Ferrous sulphate	4.5 g
Distilled water	298 ml
Concentrated hydrochloric acid	2 ml
Solution 2	
Distilled water	10 ml
Haematoxylin	100 mg

Working solution
Mix 15 ml of Solution 1 with 5 ml of Solution 2. The working solution is stable for 1 h only.

Steps

- Stain with the working solution for 7 min.
- Wash the section in distilled water.
- Differentiate in 0.2% hydrochloric acid: 5 s.
- Wash well.
- Dehydrate in acetone.
- Clean in xylene.
- Mount in DPX.

Result Phospholipid takes a blue colour.

9.6 Nucleic Acid and Proteins

9.6.1 Nucleic Acids

Nucleic acids are of two types: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA consists of two helical strands made of alternate sugar and phosphate molecules. Four types of bases are linked with each sugar molecule. These bases are purine (adenine and guanine) and pyrimidine (cytosine and thymine). Adenine only joins with thymine and cytosine only joins with guanine. This is known as complementary base pairing. There is another pyrimidine base known as uracil that is found in RNA.

The constituents of nuclei acid are:

- Sugar
- Phosphate
- Base: Purine and pyrimidine

9.6.2 Proteins

Proteins are made of amino acids. Each amino acid contains a central carbon atom with attached amino group and carboxylic group in each side (Fig. 9.9).

9.6.3 Feulgen Stain [9]

This stain is specific for DNA and it demonstrates sugar deoxyribose. This is particularly helpful for DNA ploidy examination.

Basic Principle In the presence of acidic environment (hydrochloric acid treatment), the purine bases of the DNA molecule are detached from the deoxyribose. DNA molecule becomes apurinic; however sugar-phosphate backbone of DNA is preserved. The aldehyde group of the sugar molecule is exposed, and this group subsequently binds with Schiff's reagent to impart colour. This hydrolysis part is the most vital part of this stain.

DNA + Hydrochloric acid → Exposed aldehyde group of deoxyribose

Aldehyde group + Schiff's reagent → Reddish purple colour

Application Feulgen stain is particularly helpful for DNA ploidy examination.

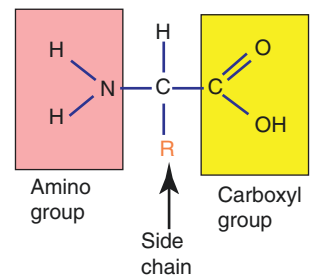

Fig. 9.9 Structure of amino acid. It contains an amino and also a carboxyl group

1M Hydrochloric acid solution

1M Hydrochloric acid	8.5 ml
Distilled water	91.5 ml

Schiff's Reagent Described previously

Potassium metabisulphite solution

10% potassium metabisulphite	5 ml
1 M hydrochloric acid	5 ml
Distilled water	90 ml

Steps

1. Rehydration of section/smear by graded alcohol.
2. Rinse in water.
3. Put in 1 (M) hydrochloric acid (preheated at 60 °C) for 60 min.
4. Keep in Schiff's reagent for 45 min.
5. Immerse in 0.05M metabisulphite for 2 min three times each.
6. Counterstain with 0.01% fast green.
7. Dehydrate in absolute alcohol.
8. Clean in xylene.
9. Mount.

Result DNA takes a reddish purple colour.

9.6.4 Methyl Green-Pyronin Stain [10]

Methyl green-pyronin stain demonstrates DNA and also RNA.

Fixation: Formalin fixation

Solution

Methyl green-pyronin (2%) in distilled water	9 ml
Pyronin Y (2%) in distilled water	4 ml
Glycerol	14 ml
Acetate buffer (pH 4.8)	23 ml

Add the ingredients and mix well.

Steps

- Deparaffinize.
- Pass through graded alcohol.
- Wash in water.
- Wash in acetate buffer.
- Keep in methyl green-pyronin solution for 30 min.
- Wash with buffer.
- Rinse in distilled water.
- Dehydrate in alcohol.
- Clean in xylene.
- Mount.

Result

DNA takes a bluish green colour.

RNA takes a red colour.

9.7 Pigments

Various organic and inorganic pigments are present in our body. The pigments may be endogenous or exogenous in origin (Table 9.4).

9.8 Hemosiderin Pigment

Hemosiderin is the breakdown product of haemoglobin.

9.8.1 Prussian Blue Reaction (Perls' Reaction) for Ferric Iron

Principle Hydrochloric acid unmasks the ferric iron. This ferric iron reacts with potassium ferrocyanide to form the insoluble blue-coloured ferric ferrocyanide.

Table 9.4 Different types of pigments

Endogenous pigment	Exogenous pigment	Artefact looking as pigments
Haematogenous • Haemosiderin • Haemoglobin • Bile pigment • Porphyrin Nonhaematogenous • Argyrophil pigments • Melanin • Lipofuscin • Dubin-Johnson • Chromaffin	• Calcium • Copper • Uric acid	• Formalin • Starch • Malaria • Schistosome

Solution

Solution A: Potassium ferrocyanide	2 g
Distilled water	100 ml
2% aqueous solution of potassium ferrocyanide	
Solution B: Hydrochloric acid	2% (2 ml hydrochloric acid and 98 ml distilled water)
Counterstain	
Neutral red	1% aqueous solution

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Dip in a mixture of equal parts of Solution A and Solution B: 30 min.
- Multiple dips in distilled water.
- Counterstain with neutral red for 15 s.
- Rinse in distilled water: 10–15 dips.
- Dehydrate in graded alcohol.
- Clean in xylene.
- Mount.

Result (Fig. 9.10)
Hemosiderin: Blue
Nuclei: Red

9.9 Bile Pigment

Bile pigments include conjugated and unconjugated bilirubin and biliverdin. Bile pigment is stained with Fouchet's stain [11].

9.9.1 Fouchet's Stain

Solution

Trichloroacetic acid	25 g
Distilled water	100 ml

Mix it well.

Ferric chloride	1 g
Distilled water	10 ml

Mix it well.

Now mix 100 ml aqueous trichloroacetic acid with 10 ml aqueous ferric chloride solution and keep the mixture in a dark bottle.

Van Gieson stain solution

Acid fuchsin	100 mg
Aqueous saturated picric acid	100 ml

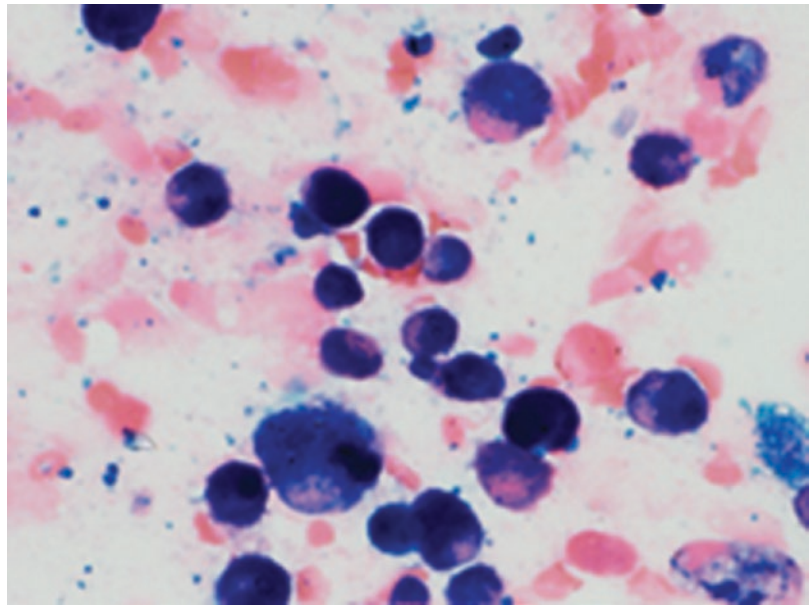

Fig. 9.10 Perls' reaction in the cytology smear shows dark blue haemosiderin pigments

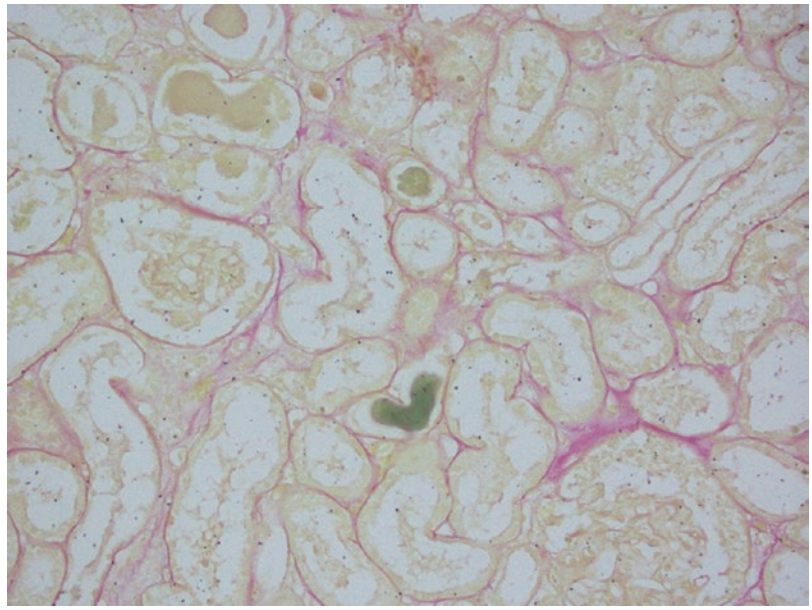

Fig. 9.11 Fouchet's stain: The stain highlights bile cast within renal tubules in a case of bile cast nephropathy (200×)

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Dip in fresh Fouchet's solution: 10 min.
- Wash in distilled water for 2 min.
- Counterstain with Van Gieson stain solution: 2 min.
- Dehydrate in graded alcohol.
- Clean in xylene.
- Mount.

Result (Fig. 9.11)
Bile pigment: Green
Collagen: Red
Muscle: Yellow

9.10 Argyrophil Pigments

9.10.1 Grimelius Staining [12]

Grimelius stain can demonstrate the argyrophil substances.

Principle

Argyrophil cells: These cells quickly absorb the silver salt, and the cells need a reducing agent to make visible silver precipitation by reduction.

Argentaffin cells: These cells also absorb the silver salt and reduce them without any external reducing substances.

Acetate Buffer

Solution 1	
Acetic acid	1.2 ml
Distilled water	100 ml
Solution 2	
Sodium acetate	2.7 g
Distilled water	100 ml

Final solution: 9 ml Solution 1 with 91 ml Solution 2, pH adjusted to 5.6

Reducing solution	
Sodium sulphite, anhydrous	5 g
Hydroquinone	1 g
Distilled water	100 ml
2% silver nitrate	
Silver nitrate	4 ml
0.2M acetate buffer, pH 5.6	10 ml
Distilled water	86 ml

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Dip the slides in silver nitrate solution (2%) in a Coplin jar: overnight.
- Drain out the silver nitrate solution.
- Keep the slides in reducing solution for 1–2 min.
- Wash in distilled water.
- Dehydrate in graded alcohol.
- Clean in xylene.
- Mount.

Result
Argyrophilic cells: Black
Background: Golden yellow

9.10.2 Melanin

Melanin is the yellowish brown to black pigment. This pigment is present in hair, the epidermis of the skin, the eye and the substantia nigra of the brain. Melanin is produced from tyrosine by series of reaction.

9.10.2.1 Masson-Fontana Method

Masson-Fontana stain demonstrates melanin and argentaffin granules.

Solution

10% Silver nitrate solution	25.0 ml

Stock solution

- Take 25 ml silver nitrate solution in a flask and add drop by drop with concentrated ammonium hydroxide.
- Shake gently.
- Add the ammonia solution until all the initial precipitate dissolves.
- Clear solution will form.

Working solution

Stock solution	12.5 ml
Distilled water	37.5 ml

Use the solution within a month.

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water for 10–15 dips.
- Dip the slides in ammoniacal silver nitrate in a Coplin jar overnight.
- Wash with distilled water three times.
- Keep in aqueous sodium thiosulphate (5%): 2 min.
- Wash thoroughly in running tap water: 2 min.
- Counterstain with neutral red (0.5% aqueous): 5 min.
- Wash in distilled water.
- Dehydrate in alcohol.
- Clean in xylene.
- Mount.

Result
Melanin, argentaffin granules, lipofuscin: Black
Nucleus: Red

9.10.3 Schmorl's Stain [13]

Schmorl's solution

Fresh solution of ferric chloride (1% aqueous)	30 ml
Fresh solution of potassium ferricyanide (0.4% aqueous)	4 ml
Distilled water	6 ml

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Now keep the section in Schmorl's solution in a Coplin jar for 15 min.
- Wash thoroughly in running tap water.
- Counterstain with neutral red (0.5%): 5 min.
- Dehydrate in alcohol.
- Clean in xylene.
- Mount.

Result (Fig. 9.12)

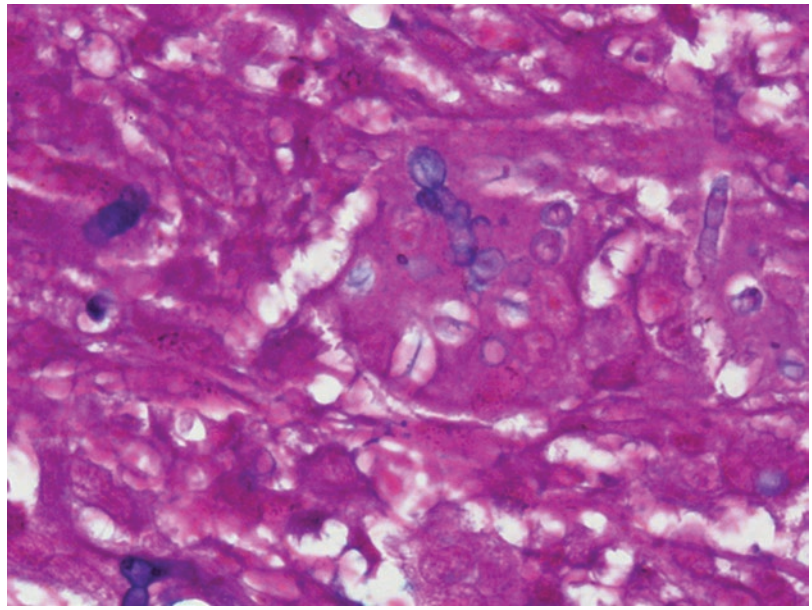

Fig. 9.12 Schmorl's stain: Microphotograph showing the hyphae of the dematiaceous fungi (phaeohyphomycosis); Schmorl's stain highlights the peacock green-coloured pigmented fungi (1000× oil immersion)

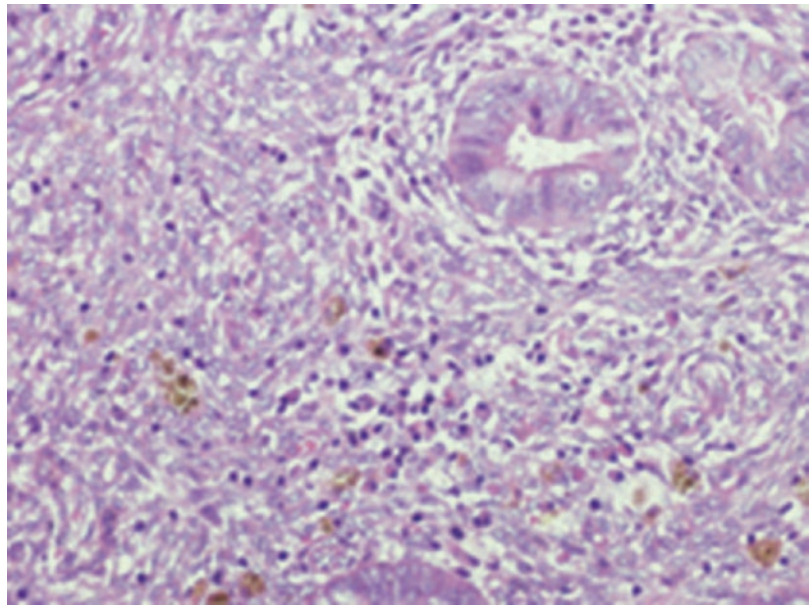

Fig. 9.13 Brownish formalin pigments in the tissue (haematoxylin and eosin ×200)

Melanin, argentaffin granules, lipofuscin: Dark blue

Nucleus: Red

9.10.4 Calcium

Inorganic calcium is the important constituents of bone and teeth. Calcium can be deposited abnormally in necrotic tissue or in case of infraction.

Von Kossa technique [14, 15]

Silver nitrate (5%)	
Silver nitrate	5.0 g
Distilled water	100.0 ml
Thiosulphate solution	
Sodium thiosulphate	5 g
Distilled water	100 ml

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Flood the section with silver nitrate: 1 h in strong sunlight.
- Wash thoroughly in running tap water.
- Keep in sodium thiosulphate solution: 5 min.
- Wash thoroughly in distilled water.
- Counterstain with neutral red (0.5%): 5 min.
- Dehydrate in alcohol.
- Clean in xylene.
- Mount.

Result
Calcium deposits: Black
Nuclei: Red

9.10.5 Formalin Pigment

Formalin pigment is brownish black in colour. Acidic formalin fixation commonly produces formalin pigment (Fig. 9.13). The pigment can be removed by alcoholic picric acid. Using buffered formalin helps to reduce formalin pigment.

9.10.5.1 Malarial Pigment

Malarial pigment is similar to formalin pigment. This is also brownish black in colour. Unlike formalin pigment, malarial pigment is intracellular in location. RBCs are usually loaded with malarial pigment. This pigment can be removed by treating the section with alcoholic picric acid.

9.10.5.2 Starch

Starch pigment is produced by talcum powder used in the gloves of the surgeons. The pigment is positive for PAS stain.

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Connective Tissue Stain: Principle and Procedure

10

Connective tissue is one of the major types of tissue that connects the different parts of tissue and also supports the body parts. Mature connective tissue is classified as:

1. Connective tissue proper:
   a. Loose areolar tissue
   b. Dense connective tissue
2. Specialized connective tissue:
   c. Adipose tissue
   d. Haematopoietic tissue
   e. Bone
   f. Cartilage

The connective tissue consists of two major elements:

- Cells
- Noncellular substances

The noncellular substances can be divided into:

- Fibrous part
- Amorphous ground substances also known as matrix

Major cells in the connective tissue are divided into:

Fixed cells (1) Fibroblasts, (2) adipose cells, (3) macrophages, (4) pericytes and (5) mast cells

Transient cells (1) Plasma cells, (2) lymphocytes, (3) neutrophils, (4) monocytes, (5) eosinophils and (6) basophils

10.1 Fibrous Part of Connective Tissue

The fibrous part of the connective tissue includes:

- Collagen
- Reticulin
- Elastin
- Basement membrane

10.1.1 Collagen

Collagen fibres are derived from the fibroblasts. Collagen fibre may remain as individual single fibre or in bunches. There are predominantly five types of collagen fibres:

Collagen I: These are 300-nm-long and much thicker fibres. They carry the main bulk of connective tissue. Collagen I fibres are present in the skin, bone, tendon and also blood vessels.

Collagen II: Collagen II fibres are composed of thin fibrils and are arranged as fine meshwork. They are present in hyaline and elastic cartilage and cornea.

Collagen III: It is present along with collagen I. The tensile strength of collagen III is much less

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than collagen I. Collagen III is present in the basement membrane of various organs.

Collagen IV: They are fine fibrillar structures that are randomly arranged. Collagen IV fibres are present in the glomerular basement membrane of the kidney.

Collagen V: This is a fibril-forming collagen and is present in minor quantities in specific tissue such as placenta and atherosclerotic plaque. It is also present in the interstitial tissue of the kidney and inter-alveolar septum of the lung.

Collagen VI: This is a unique member of collagen family. Collagen VI is connected with various components of extracellular matrix material.

Table 10.1 highlights the different types of collagen.

Table 10.1 Comparison of the different types of collagen

Types of collagen	Fibrillary pattern	Location	Function
I	Fibrillar, thicker	Skin, bone, dentin, tendon	Gives tensile strength
II	Fibrillar, thin	Cartilage	Gives tensile strength
III	Fibrillar, thin, associated with type I collagen	Blood vessels, lymph node, lung	Provides structural framework of the lymph node and spleen and gives tensile strength to various connective tissues
IV	Network forming	Basement membrane	Makes the framework of lamina densa to provide support
V	Fibrillar	Present along with type I, placenta, interstitial tissue of the kidney and inter-alveolar septum of the lung	Gives tensile strength
VI	Beaded filaments	Connective tissue of the blood vessels, uterus, etc. present along with type II	Possibly helps in the attachment of cells and connective tissue

10.1.2 Reticulin Fibres

Reticulin fibres are the fine branching fibres that are interwoven within closely. They are actually collagen III fibre. These are argyrophilic fibres. Reticulin fibres support the parenchymal tissue of the liver, spleen and lymph node.

10.1.3 Elastic Fibres

These are fine fibres. They are present as branching fibres or sheet. The elastic fibres are made of microfibrils that are organized in complex pattern with the help of calcium. Myofibrils of the elastic fibres interact with various proteoglycans that help in the integration of the supporting tissue. Elastic fibres provide the elasticity of the blood vessels, lung and skin.

10.1.4 Basement Membrane

Basement membrane is the connective tissue elements that separate the epithelial and endothelial cells from the underlying connective tissue. The basement membrane is divided into three layers from cell membrane to away:

1. Lamina lucida: This is nearer to the surface cells and is made of various carbohydrate materials. The lamina lucida contains integrin, laminin and collagen. This layer may be simply an artefact.
2. Lamina densa: This is the next zone of basement membrane and consists of predominantly collagen IV, proteoglycan, laminin and fibronectin.
3. Lamina fibroreticularis: This is the fibrous component and merged with the underlying connective tissue elements. They are composed of bunch of microfibrils and collagen fibres.

10.2 Stains

10.2.1 Masson Trichrome [1]

Many different colours of dye are used in Masson trichrome stain to differentiate the collagen fibres, muscle, fibrin and RBCs.

Indications:

- Liver biopsy: To assess the degree of fibrosis in the liver tissue such as in chronic liver disease, cirrhosis, etc.
- Kidney biopsy: To see the changes of basement membrane of glomeruli
- Tumour: To distinguish collagen and smooth muscle in tumours

Principle The staining of a particular tissue by a particular dye depends on the size, molecular weight, ionic character and network of the molecules of the tissue. Similarly the molecular weight and ionic character of the dye are important. Dye of lower molecular weight will penetrate the tissue more easily and will stain all the components of the tissue. The dye of medium molecular weight will penetrate only through the muscle and collagen (leaving RBC uncoloured), whereas the more higher molecular dye will penetrate only through collagen, and muscle fibres will not be stained at all. So the basic thing is that the smaller dye molecule will stain the tissue, and when the larger dye molecule penetrates the tissue, then it will replace the smaller dye from the tissue.

In Masson trichrome stain, the dyes of different molecular weights are used in sequential manner.

Acid fuchsin dye is used to stain both muscle and collagen tissue together. The staining time is kept for optimum duration so that the dye stains the muscle and collagen adequately.

Then, a polyacid of larger molecular size is used to differentiate the tissue. It removes the stain from the bone and collagen.

Finally, another fibre-specific contrast dye (aniline blue) is used that has larger molecular weight than the previous (acid fuchsin) dye. This dye is kept for optimum time to stain the collagen fibres only.

Solution

1. Bouin's fixative	
Saturated picric acid	75 ml
Formaldehyde (40%)	25 ml
Glacial acetic acid	5.0 ml

2. Weigert's haematoxylin	
Solution (a)	
Haematoxylin	1.0 g
95% ethanol	100 ml
Solution (b)	
29% aqueous ferric chloride	4.0 ml
Distilled water	95.0 ml
Concentrated hydrochloric acid	1.0 ml
Working solution: Mix equal proportion of solutions (a) and (b), and use immediately	
3. Acid fuchsin solution	
Acid fuchsin	0.5 g
Glacial acetic acid	0.5 ml
Distilled water	100 ml
4. Phosphomolybdic acid solution (1%)	
Phosphomolybdic acid	1 g
Distilled water	100 ml
Mix phosphomolybdic acid in distilled water. This solution is stable for 6 months	
5. Aniline blue solution	
Aniline blue	2.5 g
Glacial acetic acid	2.0 ml
Distilled water	100 ml

Preparation: Mix aniline blue in 100 ml of boiling distilled water. Mix 2 ml glacial acetic acid. Cool the solution and then filter.

Steps to stain

- Deparaffinize.
- Graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Fix the section in Bouin's fixative: 60 min.
- Rinse thoroughly in running tap water: 10 min.
- Weigert's iron haematoxylin: 10 min.
- Bluing: by keeping the section in running tap water.
- Acid fuchsin: 10 min.
- Rinse with acetic acid.
- Treat with phosphomolybdic acid solution: 5 min.
- Drain the solution.
- Aniline blue: 5 min.
- Wash in distilled water: 10–15 dips.
- Differentiate: 2% acetic acid—2 min.
- Wash in distilled water.
- Rapid dehydration.
- Clear in xylene.
- Mount.

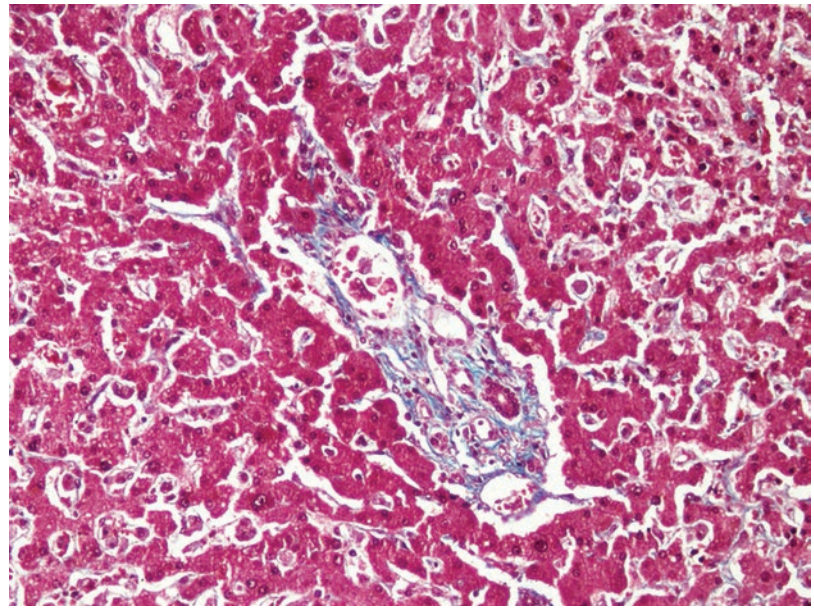

Fig. 10.1 Masson trichrome (MT) stain: the stain highlighting the portal tract bluish green, while the hepatocytes were stained red. The nuclei of the cells took a deeper shade of blue (normal liver tissue) (200×)

Result (Fig. 10.1)
Muscle: red
Collagen: blue
Nuclei: black or blue
Fibrin: red

10.2.2 Van Gieson Stain [2]

Aim This stain demonstrates collagen.

Indications:

- Extent of fibrosis: It helps to assess the quantitation of fibrosis.
- Tumour: It helps to differentiate collagenous material from the smooth muscle so useful to differentiate fibrosarcoma from leiomyosarcoma.
- Amyloid versus collagen: Van Gieson stain is specific for collagenous material.

Principle The principle of this stain is the same as described in Masson trichrome stain.

Van Gieson's stain solution

Acid fuchsin 1% (aqueous)	10 ml
Picric acid (aqueous saturated)	100 ml

Steps of staining

- Deparaffinize.
- Graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Weigert's iron haematoxylin: 10 min.
- Wash in tap water.
- Differentiation: 1% acid alcohol.
- Wash in water.
- Van Gieson's solution: 5 min.
- Wash in distilled water.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Result (Fig. 10.2)
Collagen fibres: red
Nuclei: black
Cytoplasm, muscle and fibrin: yellow

10.2.3 Reticulin Stain

Indications:

- Liver biopsy: Reticulin stain helps to demonstrate the early cirrhosis. It is an essential stain in liver sections.

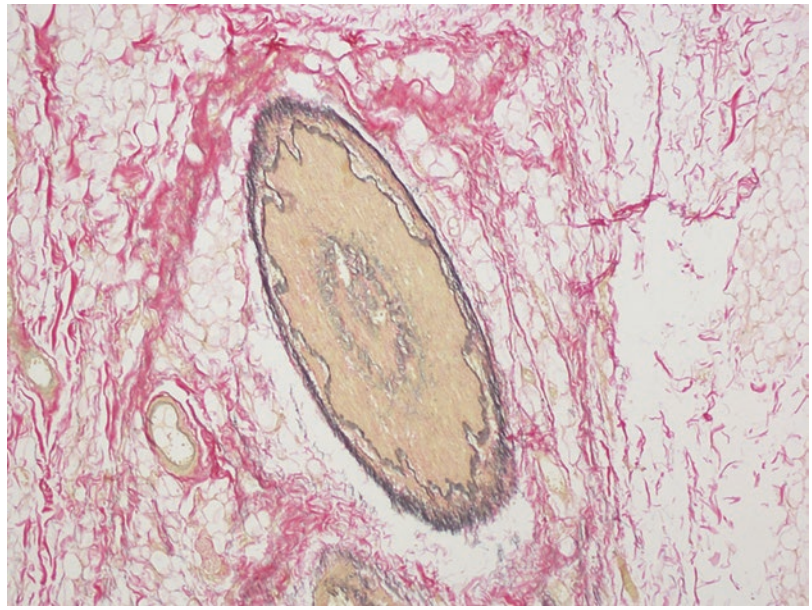

Fig. 10.2 Elastic van Gieson (EVG) stain: elastic van Gieson staining in a case of polyarteritis nodosa. The elastic tissue of the artery (elastic lamina) was stained black, while the fat was stained red (200×)

- Kidney: It demonstrates Kimmelstiel-Wilson lesion of diabetic glomerulosclerosis.
- Bone marrow: It is a very useful stain to demonstrate marrow fibrosis and is particularly helpful in myelofibrosis.
- Lymph node: Lymph nodal architectural pattern is better demonstrated by reticulin stain. The loss of lymph nodal architecture is seen in non-Hodgkin lymphomas.
- Tumours:
  - In endometrial stromal sarcoma, "basket-weave" appearance is seen.
  - Chicken wire pattern of reticulin is noted in myxoid liposarcomas.
  - Typical Zellballen pattern in paraganglioma is better demonstrated by reticulin stain.
  - Gliomas and sarcomas: Abundant reticulin fibres
  - Ewing's sarcoma: Absent of reticulin

Principle Reticulin fibres are stained reliably by silver impregnation method (Fig. 10.3). The reticulin fibres contain a carbohydrate component. Potassium permanganate is used to oxidize the reticulin fibres, and aldehyde group is generated from the carbohydrate component. The tissue is treated with silver salt in basic pH. In the basic medium, silver salt produces metallic silver that reacts with the aldehyde group of the tissue. Subsequently sodium thiosulphate is used to remove the excess unreactive silver. Tissue is further treated with gold chloride to make this precipitation permanent.

10.2.4 Gordon and Sweet's Method for Reticulin Stain [3]

10.2.4.1 Solution

Acidified Potassium Permanganate (1%)

• Potassium permanganate	0.5 g
• 3% sulphuric (H_2SO_4) acid	2.5 ml
• Distilled water	47.5 ml
Freshly prepared solution	

Oxalic Acid (2%)

Dissolve 2.0 g oxalic acid in 100 ml distilled water.

Iron Alum (2%)

Ferric ammonium sulphate	2.0 g
Distilled water	100.0 ml

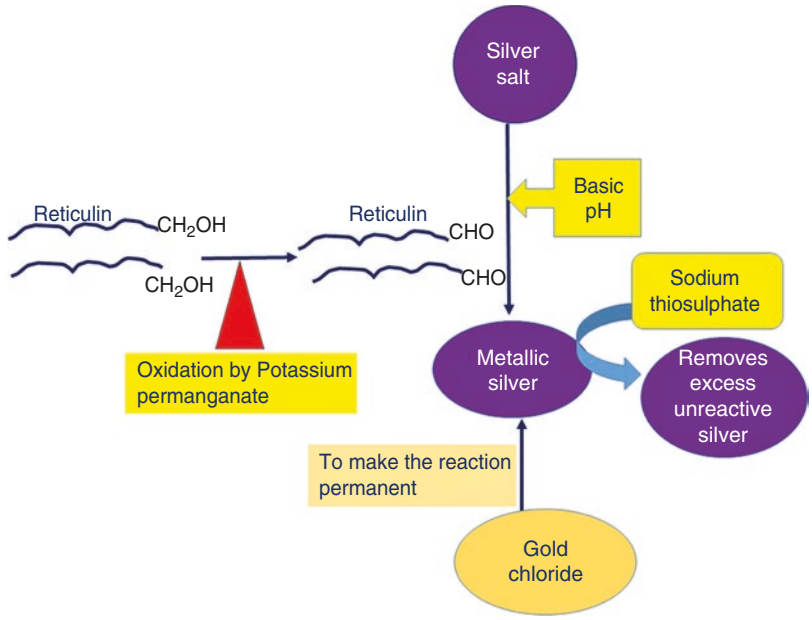

Fig. 10.3 Schematic diagram shows mechanism of reticulin stain. Potassium permanganate oxidizes the carbohydrate component of the reticulin fibres to generate aldehyde group. In the basic medium, the silver salt produces metallic silver that reacts with the aldehyde group of the tissue. Gold chloride makes this metallic precipitation permanent. In addition, sodium thiosulphate is used to remove the excess unreactive silver

10% Formaldehyde

Formaldehyde	10.0 ml
Distilled water	90 ml

Gold Chloride (0.2%)

Dissolve 0.2 g gold chloride in 100 ml distilled water.

Sodium Thiosulphate (5%)

Sodium thiosulphate	5 g
Distilled water	100.0 ml

Silver Nitrate (10%)

Silver nitrate	10.0 g
Distilled water	100.0 ml

Take 5 ml aqueous silver nitrate solution, and add drop by drop sodium hydroxide. Initial precipitate will be formed. Add strong ammonia drop by drop to dissolve the black precipitate. Now add equal amount of distilled water.

Steps to stain

- Deparaffinize.
- Graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Potassium permanganate solution: 5 min.
- Wash in tap water.
- Bleach: Oxalic acid 2 min.
- Wash in tap water.
- Iron alum solution, 10–15 min.
- Wash in distilled water three changes.
- Silver nitrate solution: 2 min.
- Wash in distilled water two changes.
- 10% formaldehyde solution: 1 min (till it becomes grey black).
- Rinse thoroughly in tap water.
- Toning: 0.2% gold chloride solution for 3 min.
- Rinse thoroughly in tap water.
- Fix with sodium thiosulphate (5%): 2 min.
- Wash in tap water.
- Counterstain: Neutral red for 2 min (if you need).
- Wash in distilled water.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Result (Fig. 10.4)
Reticulin fibres: black
Nuclei: red
Warning notes

- Silver salt solution should be properly prepared. Do not mix excess ammonia solution in the silver nitrate.
- Use a coplin jar for the silver nitrate solution treatment. Time is a critical factor in this step

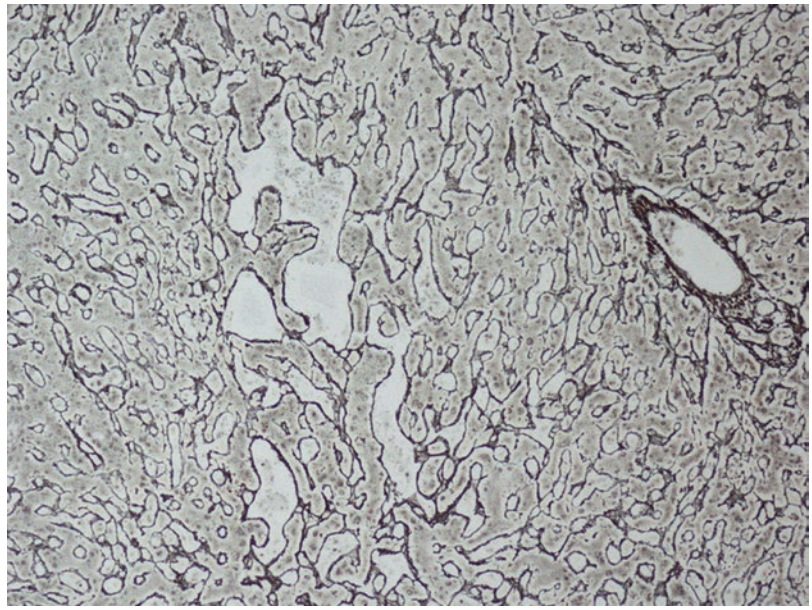

Fig. 10.4 Reticulin stain: reticulin stain in the liver highlighting the normal reticulin pattern of the liver (400×)

of silver salt reaction. Do not put the slide in silver salt solution for excess time.
- Silver salt solution should be away from sunlight.
- Use only distilled water in each step.
- Clean glassware should be used. Wash them by distilled water thoroughly, at least five times.

10.3 Elastic Fibres

The common stains of elastic fibres include Verhoeff's stain, orcein stain and Weigert's resorcin-fuchsin stain.

10.3.1 Verhoeff's Stain for Collagen [4]

Principle The haematoxylin dye binds with the elastic tissue by ionic interaction. Ferric salt acts as an oxidizer and helps in binding of haematoxylin and elastic fibres.

Control: skin tissue

Solutions

Solution 1	
Haematoxylin	5 g
Absolute alcohol	100 ml

It is preferable to use fresh solution	
Solution 2	
Ferric chloride	10 g
Distilled water	100 ml
Solution 3	
Iodine	1 g
Potassium iodide	2 g
Distilled water	100 ml
Final Verhoeff's solution	
Solution 1	20 ml
Solution 2	8 ml
Solution 3	8 ml

Add 8 ml "solution 2" into 20 ml of solution 1 and then add 8 ml solution 3.

Steps of staining

- Deparaffinize.
- Graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Verhoeff's iron haematoxylin: 20 min.
- Differentiation: By 2% ferric chloride. This is a crucial step. Allow to differentiate till the fibres take black colour. Check the colour by microscope.
- Wash in water.
- Remove iodine by washing in 95% alcohol.
- Counterstain: Van Gieson's stain—2 min.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Results
Elastic fibres: black
Nuclei: black
Collagen: red
Other tissue: yellow

10.3.2 Weigert's Resorcin-Fuchsin Stain [5]

Solution

Basic fuchsin	2 g
Resorcin	4 g
Distilled water	200 ml

- Mix basic fuchsin and resorcin in 200 ml distilled water and boil. Now add 25 ml of 30% ferric chloride in this boiling solution.
- Boil for another 5 min
- Cool
- Filter
- Take the precipitate in a filter paper
- Now take 200 ml of 95% ethanol in a flask and dissolve the precipitate by heating
- Remove the filter paper
- Add 4 ml of concentrated HCl

Steps of staining

- Deparaffinize.
- Graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Put the section in the stain solution for 1–3 h in room temperature.
- Wash in water.
- Differentiate: by 1% acid alcohol.
- Wash in water.
- Counterstain: Eosin.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Result
Elastic fibres: purple

10.3.3 Orcein for Elastic Fibres

Orcein	1 g
Hydrochloric acid	1 ml
70% alcohol	100 ml

- Dissolve orcein in alcohol and heat the solution
- Cool
- Filter
- Add hydrochloric acid

Steps of staining

- Deparaffinize.
- Graded alcohol to bring in water.
- Wash in water.
- Orcein solution: 30 min (in 56 °C).
- Differentiate: by 1% acid alcohol.
- Wash in water.
- Counterstain: methylene blue.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Result
Elastic fibres: brown
Fibrin and cross striation of the muscle
Fibrin is best stained by phosphotungstic acid haematoxylin (PTAH).

10.3.4 Phosphotungstic Acid Haematoxylin (PTAH) [6, 7]

Aim PTAH aims to stain fibrin, cross striation of the muscle and glial fibres.

Solutions

Haematoxylin	1 g
Phosphotungstic acid	20 g
Distilled water	1000 ml

- Dissolve haematoxylin and phosphotungstic acid separately in distilled water by gently heating.
- Cool.
- Combine the two solutions.

0.25% potassium permanganate

Potassium permanganate	0.25 g
Distilled water	100 ml

5% oxalic acid

Oxalic acid	5 g
Distilled water	100 ml

Steps of staining

- Deparaffinize.
- Graded alcohol to bring in water.
- Wash in water.
- Oxidation: by 0.25% potassium permanganate for 10 min.
- Wash in water.
- Bleaching: by 5% oxalic acid for 2–5 min (until the colour disappears).
- Wash thoroughly in distilled water.
- Keep the section in PTAH solution for 12–24 h.
- Dehydration: rapidly by 95% ethyl alcohol.
- Absolute alcohol.
- Clear.
- Mount.

Result (Fig. 10.5)

Striated muscle fibres, fibrin, nuclei and astrocytes: blue

- Cytoplasm: brown red
- Collagen and bone: brown pink

Figure 10.6 demonstrates different staining pattern for different types of connective tissue.

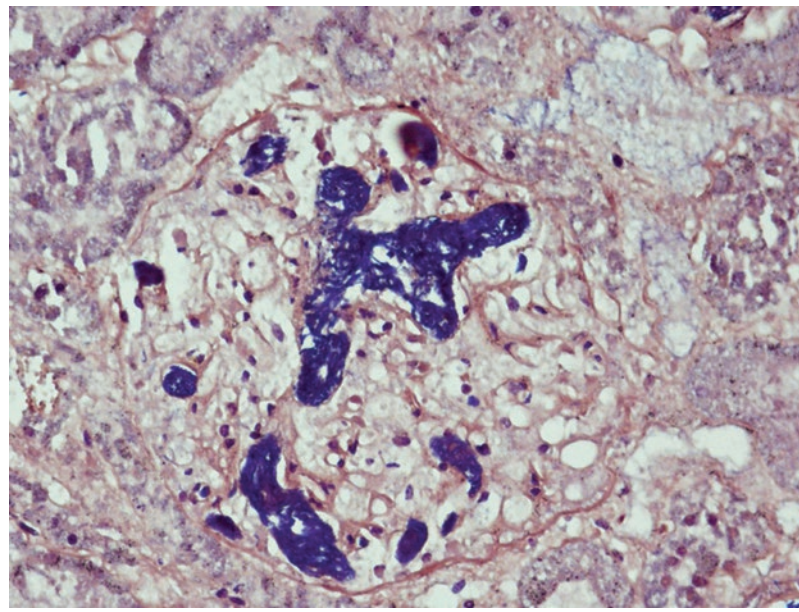

Fig. 10.5 Phosphotungstic acid haematoxylin (PTAH) stain: This stain highlighted the fibrin thrombi as haematoxyphilic blue showing numerous glomerular fibrin thrombi (400×)

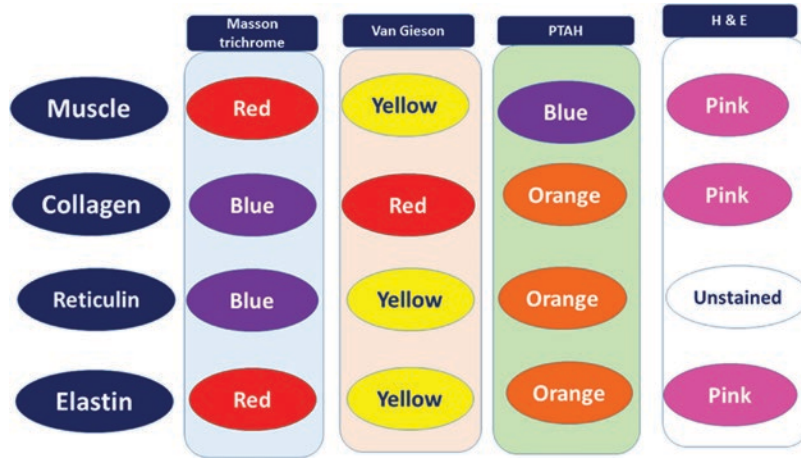

Fig. 10.6 Staining the colour pattern of different connective tissue stains (*PTAH* phosphotungstic acid haematoxylin, *H&E* haematoxylin and eosin)

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Amyloid Staining

11

11.1 Introduction

Amyloid is the eosinophilic amorphous extracellular insoluble misfolded fibrillar protein. The deposition of this extracellular amyloid protein in various organs and tissues is known as amyloidosis. Amyloid is not a single disease. The different diseases of variable etiopathogenesis may show amyloidosis.

Amyloid protein is the beta-fibrillar structure which gives the characteristic staining pattern. Amyloid deposit is composed of fibrillar amyloid protein, plasma, proteoglycan and extracellular matrix material. On electron microscopy, the amyloid fibril shows non-branching filaments that are randomly arranged. The amyloid fibrils are specifically arranged as beta-pleated sheet configuration which give their characteristic tinctorial pattern. Each fibril is composed of two or more subunits. Each filamentous subunit is 2.5–3 nm in diameter and in total 10 nm in diameter. The fibrillar subunits are parallel and twisting with each other forming a beta-pleated sheet. They are folded and arranged in antiparallel orientation with the adjacent polypeptide chains. The stability of the beta-pleated sheet is a critical factor for the further deposition of the amyloid protein irrespective of the chemical characters of the protein.

Amyloid is classified as systemic amyloidosis or localized amyloidosis. The systemic amyloidosis is characterized by deposition of amyloid in multiple organs, whereas localized amyloidosis shows only single organ involvement [1–3].

The systemic amyloidosis is classified broadly as (Table 11.1):

1. Primary amyloidosis
2. Secondary amyloidosis
3. Familial amyloidosis
4. Other familial types of amyloidosis

Primary amyloidosis:

AL type amyloidosis (AL): AL amyloidosis is associated with plasma cell neoplasm, and it is the commonest type of amyloidosis. The fibril composition of AL type is variable region of

Table 11.1 Classification of amyloidosis

Type	Biochemical variety
Systemic	
Primary: related with plasma cell tumour	AL
Secondary: related with chronic diseases	AA
Haemodialysis related	Aβm
Heredofamilial	
Hereditary polyneuropathy	ATTR
Familial Mediterranean fever	AA
Localized	
Senile cardiac	ATTR
Senile cerebral	Aβ
Endocrine: medullary carcinoma of thyroid	A Cal

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kappa or lambda light chain of immunoglobulin. The free light chain is present in circulation and is also deposited in the various organs. The exact cause of amyloidogenic potential of the light chain is not surely known possibly it depends on certain sequence of amino acid.

Secondary amyloidosis (AA type):

The fibrillar composition of secondary amyloidosis is AA protein. This type of amyloidosis is secondary to various inflammatory lesions such as tuberculosis, rheumatoid arthritis, etc. The amyloid is formed from serum amyloid A (SAA), an acute phase protein.

Familial amyloidosis (ATTR)

In this autosomal dominant disorder, the amyloid is formed from the mutant transthyretin (ATTR). Transthyretin is a transport protein of thyroxine- and retinol-binding protein.

Localized amyloidosis

In case of localized amyloidosis, the amyloid is deposited in the isolated organ such as the lung, kidney or tongue.

11.2 Stains for Amyloid

11.2.1 Alkaline Congo Red Stain [4]

Principle Congo red intercalates between the parallel fibrils of the amyloid protein and forms a non-polar hydrogen bond.

Solution

1% sodium hydroxide	
Sodium hydroxide	1 g
Distilled water	100 ml
Saturated sodium chloride in ethanol (80%)	
80% ethanol	100 ml
Sodium chloride	1 g
Alkaline alcohol sodium chloride solution	
Sodium hydroxide (1%)	1 ml
Sodium chloride ethanol (80%)	100 ml
Freshly made solution should be used	
Alkaline Congo red stock solution	
Congo red	0.5 g
Alkaline alcohol sodium chloride solution	300 ml
Working solution of Congo red	
Alkaline Congo red stock solution	100 ml
1% sodium hydroxide	1 ml
Use only fresh solution	

Steps of staining

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Stain by Mayer's haematoxylin.
- Blueing by running tap water.
- Wash in distilled water.
- Alkaline alcohol sodium chloride solution: 20 min.
- Alkaline Congo red: 20 min.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Result (Fig. 11.1):
Amyloid: Deep pink
Apple green birefringence in polarized light

11.2.2 Congo Red Stain by Highman [5]

Congo red solution

50% ethyl alcohol	100 ml
Congo red	0.5 g
Potassium hydroxide (0.2%)	
80% ethyl alcohol	100 ml
Potassium hydroxide	0.2 g

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Congo red: 5 min.
- Differentiation by alcoholic potassium hydroxide: Few seconds.
- Wash in water.
- Counterstain: Alum haematoxylin.
- Running tap water for blueing.
- Wash by distilled water.
- Rapid dehydration.
- Clear in xylene.
- Mount.

Result
Amyloid: Red colour

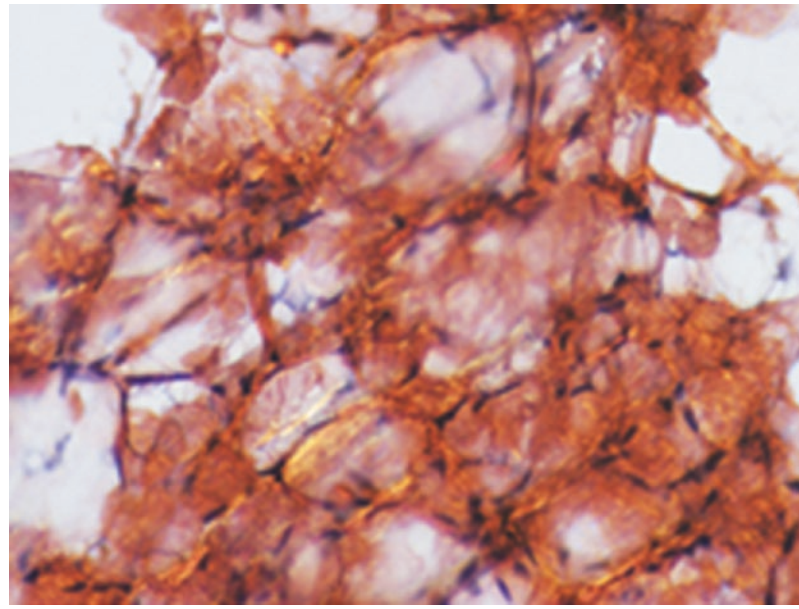

Fig. 11.1 Apple green birefringence of amyloid material in Congo red stain with polarized microscope

11.2.3 Thioflavine T Stain [6]

Thioflavine T is a very sensitive technique. However, it is not a specific stain for amyloid.

Thioflavine T Solution

Thioflavine T	1 g
Distilled water	100 ml

Steps

- Deparaffinize.
- Pass through graded alcohol to bring in water.
- Rinse in distilled water: 10–15 dips.
- Counterstain with alum haematoxylin: 2 min.
- Wash in water.
- Thioflavine T solution: 3 min.
- Rinse in water.
- Differentiation: 1% acetic acid for 20 min.
- Wash in water.
- Dehydrate.
- Clear in xylene.
- Mount in glycerine jelly.

Result

Fluorescence microscope using BG12 exciter filter and K 530 barrier filter: Bright yellow fluorescence

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Stains for the Microbial Organisms 12

Microbial organisms or microbes are submicroscopic infective organisms that may produce disease in human.

Broadly these microbes can be subdivided into (1) bacteria, (2) fungi, (3) protozoa, (4) helminths and (5) virus.

Routine haematoxylin and eosin stain may not distinctly identify the microbial organism in tissue or smear, and therefore special stain is needed. Special stain helps to delineate the morphology and characteristic colour of the organisms [1–3] (Table 12.1). It is advisable to have culture of the microbes if possible.

12.1 Bacteria

The common stains of the demonstration of bacterial organisms are:

- Gram's stain
- Ziehl-Neelsen stain

12.1.1 Gram's Stain [4, 5]

This stain is discovered by Hans Christian Gram in 1884. It helps to identify the Gram-positive organism.

Principle The Gram-positive bacteria contain good amount of peptidoglycan on their cell wall that helps to retain the dye crystal violet on the cell wall. Now Gram's iodine is added that helps to bind the crystal violet on the wall of the bacteria. This dye-iodine complex does not diffuse out in the presence of acetone. Therefore, Gram-positive organism shows blue colour even after acetone treatment. The dye-iodine complex is diffused out easily from the Gram-negative bacteria and so it becomes colourless. The counterstain carbol-fuchsin is applied to give Gram-negative organism a pink colour.

Reagents

Crystal violet solution	
Crystal violet	1.0 g
Absolute alcohol	20 ml
Ammonium oxalate (1%)	80 ml
Lugol's iodine	
Iodine crystal	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml
Basic fuchsin	
Basic fuchsin	1.0 g
Distilled water	100 ml

Steps of staining

- Deparaffinize.
- Pass through graded lower concentration of alcohol and section/smear to bring in water.
- Crystal violet solution: 1–2 min.
- Lugol's iodine: 1 min.
- Differentiation by alcohol: 1 or 2 dip.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_12

Table 12.1 Special stains for microbial organisms

Organisms	Stain	Comment
Bacteria		
Mycobacterium tuberculosis	Ziehl-Neelsen (Z-N) stain, auramine-rhodamine stain	Red bacilli in Z-N stain yellow fluorescence in auramine-rhodamine stain
Mycobacterium leprae	Fite acid-fast stain	Red bacilli
Actinomycosis	Papanicolaou's stain, May Grunwald Giemsa, Gomori methenamine silver	Clustered bacilli with sunray appearance
Nocardia	MGG, Gram stain, modified acid-fast stain	Thin slender long bacilli
Helicobacter	MGG, H&E and Gram stain	Flying birds like bacilli in rows
Fungi		
Candida	Papanicolaou's stain, May Grunwald Giemsa, Gomori methenamine silver, PAS	Thin slender pseudohyphae and spores
Aspergillus	PAP, PAS, H&E and GMS	Slender bacilli with acute angle branching
Mucormycosis	PAP, H&E and GMS	Broad, wide-angle branching, nonseptate
Cryptococci	Mucicarmine, Masson-Fontana silver stain	Small 3–5 μm round structure
Histoplasma capsulatum	GMS, PAS	Small 2–3 μm round structure
Pneumocystis jirovecii	GMS	
Parasites		
Amoeba (*E. histolytica*)	PAS, H&E, iron haematoxylin stain	Round-shaped small organisms
Giardia lamblia	H&E	Pear-shaped small organisms
Echinococcus granulosus	H&E, GMS, PAS	

H&E haematoxylin and eosin, *PAP* Papanicolaou, *GMS* Gomori methenamine silver, *Z-N* Ziehl-Neelsen, *PAS* periodic acid-Schiff

- Counterstain by basic fuchsin: 2 min.
- Wash with water.
- Dehydrate by absolute alcohol.
- Clear in xylene.
- Mount.

Result

Gram-negative bacteria: Purple
Gram-positive bacteria: Pink

12.1.2 Ziehl-Neelsen Stain

Aim Ziehl-Neelsen stain demonstrates acid-fast tubercular bacilli.

Principle The organisms are stained with basic fuchsin dye by heating. They resist decolourization by acid alcohol (1%). The methylene blue counterstain is used to stain decolourized non-acid-fast bacilli.

Reagents

Carbol-fuchsin	
Basic fuchsin	0.5 g
Absolute ethyl alcohol	5 ml
5% phenol (aqueous)	100 ml
Methylene blue	
Methylene blue	1.4 g
95% ethyl alcohol	100 ml
Acid alcohol	
Hydrochloric acid	10 ml
Alcohol	1000 ml

Steps of staining

- Deparaffinize.
- Pass through graded lower concentration of alcohol and section/smear to bring in water.
- In hot carbol-fuchsin solution for 30 min.
- Thoroughly washing in water.
- Differentiation: By acid alcohol till the tissue is pale.
- Counterstain: By methylene blue for 15 s.

- Wash in water.
- Dehydrate by absolute alcohol.
- Clear in xylene.
- Mount.

Result (Fig. 12.1):

- Acid-fast bacilli: Red colour
- Nuclei: Blue colour

12.1.3 Fite Acid-Fast Stain for Leprosy [6]

Aim Fite acid-fast stain helps to demonstrate mycobacterium leprosy.

Principle *Mycobacterium leprae* bacilli are weak acid fast organisms, and therefore weak concentration of sulphuric acid (5%) is used.

Methylene blue	
Methylene blue	1.4 g
95% ethyl alcohol	100 ml
Carbol-fuchsin	
Basic fuchsin	0.5 g
Absolute alcohol	5 ml
5% phenol (aqueous)	100 ml
Sulphuric acid (5%)	
Sulphuric acid	5 ml
25% ethyl alcohol	95 ml
Xylene in peanut oil solution	
Xylene	50.0 ml
Peanut oil	50.0 ml

This solution can be used for 1 year.

Steps of staining

- Deparaffinize: Keep the section in xylene/peanut oil solution for 2 min.
- Blot the excess oil.
- Wash in water: 5 min.
- Put the slide in carbol-fuchsin solution for 30 min (room temperature).
- Rinse in water: 5 min.
- Decolourize: By 5% sulphuric acid.
- Wash in water.
- Counterstain: By methylene blue for 1 or 2 dips.
- Wash in water: 5 min.
- Blot the section.
- Dry.
- Clear in xylene.
- Mount.

Result

Acid-fast bacilli and *Mycobacterium leprae* (Fig. 12.2): Red

Background: Blue

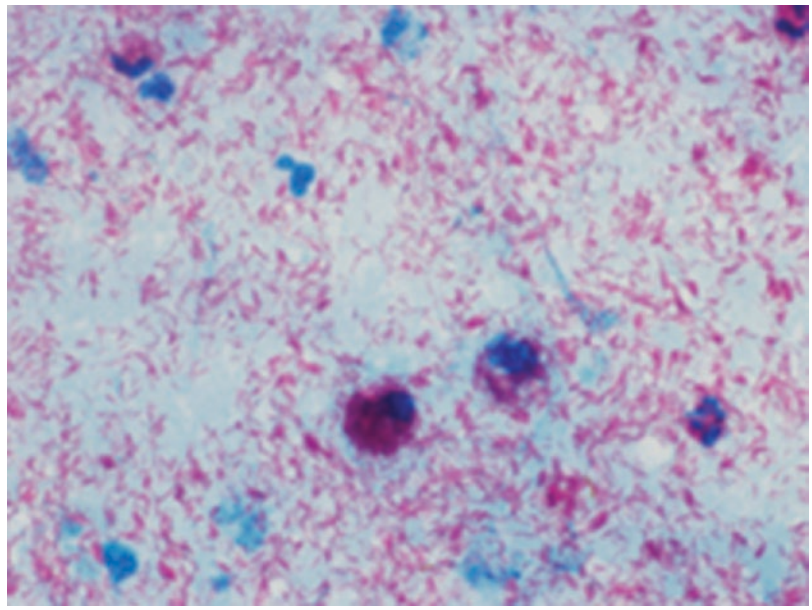

Fig. 12.1 Abundant acid-fast bacilli in a case of atypical mycobacteria (Ziehl-Neelsen stain ×1200)

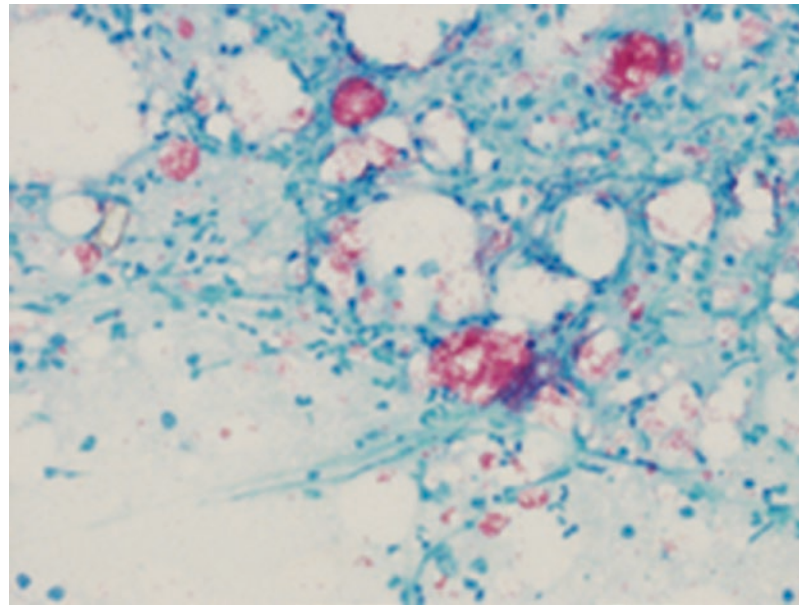

Fig. 12.2 Abundant lepra bacilli in a lymph node aspirate in Fite acid-fast stain for leprosy (Fite acid-fast stain for leprosy ×1200)

12.2 Fungal Infection

12.2.1 Grocott's Methenamine Silver [7]

Aim Grocott's methenamine silver stains the cell wall of the fungi. It stains the outer wall of the *Pneumocystis carinii* organisms.

Reagents

Stock solution of methenamine silver	
Methenamine (3%)	100 ml
Silver nitrate (5%)	5 ml

Add silver nitrate solution slowly drop by drop. The white precipitate will appear. It will dissolve slowly. Filter the solution. Keep the solution in a brown coloured bottle (stable for 3 months).

Sodium borate solution (5%)	
Sodium borate	5.0 g
Distilled water	100.0 ml

This solution can be used for 3 months.

Methenamine silver working solution	
Stock solution of methenamine silver	50 ml
5% sodium borate	5 ml

Sodium thiosulphate solution (5%)	
Sodium thiosulphate	1.0 g
Distilled water	100.0 ml
Sodium bisulphite (1%)	
Sodium metabisulphite	1.0 g
Distilled water	100.0 ml
Chromic acid (2%)	
Chromium trioxide	10.0 g
Distilled water	500.0 ml

This solution can be used for 6 months.

Gold chloride solution (0.1%)	
Gold chloride	0.1 g
Distilled water	100.0 ml
Stock solution of light green (0.2%)	
Light green	0.2 g
Distilled water	100.0 ml
Glacial acetic acid	0.2 ml
Light green working solution	
Light green stock solution	10 ml
Distilled water	50.0 ml

Steps of staining

- Deparaffinize.
- Pass through graded lower concentration of alcohol and section/smear to bring in water.
- Oxidation: 2% chromic acid, 30 min.
- Wash in distilled water.

- Dip: 1% sodium bisulphite for 1 min.
- Wash in distilled water.
- Working methenamine silver solution: 15 min in water bath at a temperature of 60 °C. Tissue will take brown colour.
- Wash in distilled water.
- Tone: Gold chloride solution 5 s.
- Wash in distilled water.
- Sodium thiosulphate solution: 5 s.
- Wash in water.
- Counterstain: Light green working solution for 10 s.
- Dehydrate by absolute alcohol.
- Clear in xylene.
- Mount.

Result (Fig. 12.3)
Fungi and pneumocystis: Black
Background: Green

12.3 Spirochaetes

12.3.1 Warthin and Starry Technique [8]

Fixation Formalin-fixed section

Reagents

Buffer solution	
Sodium acetate	1.64 g
Acetic acid	2.5 ml
Distilled water	200 ml
Silver solution	
Silver nitrate	0.5 g
Buffer solution	50 ml
Developer solution	
Solution A	
Hydroquinone	300 mg
Buffer solution	10 ml (as prepared above)
The solutions are stored in 37 °C	
Solution B	
Silver nitrate (2%)	3 ml

Mix 10 ml solution A and 3 ml solution B (prewarm to 60 °C) just before use.

Steps

- Deparaffinize and bring the section in water.
- Wash in buffer solution.
- Stain in 1% silver nitrate solution at 60 °C: 1 h.
- Keep the section in freshly prepared developer solution at 60 °C: 3–4 min.
- Wash in water at 60 °C.
- Wash in buffer solution.
- Dehydrate.
- Clear.
- Mount.

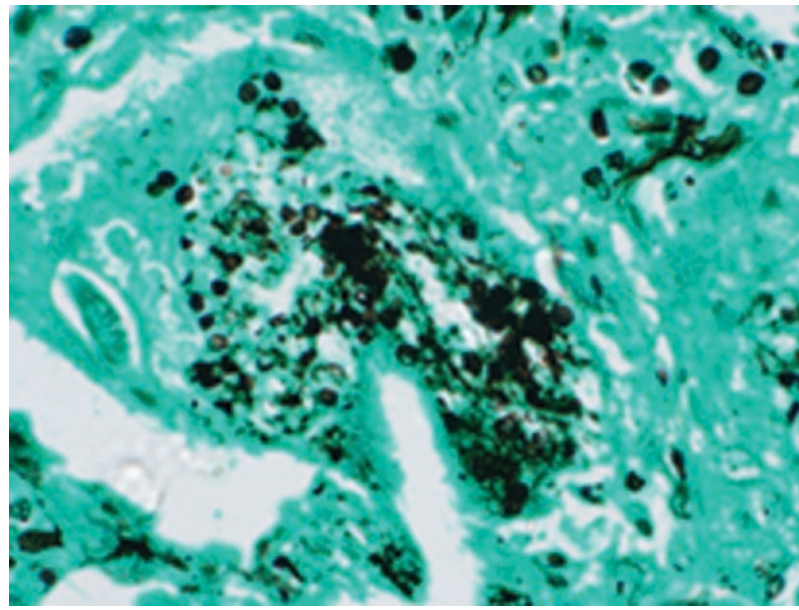

Fig. 12.3 Methenamine silver stain for *Pneumocystis carinii* infection (Methenamine silver stain ×1200)

Result

Spirochaetes: Black colour. Background: Brown to yellow

12.4 Viral Inclusions

12.4.1 Phloxine-Tartrazine Stain

Reagents

Phloxine	0.5 g
Calcium chloride	0.5 g
Distilled water	100 ml
Tartrazine to saturate in 2-ethoxy ethanol	100 ml

Steps of staining

- Deparaffinize.
- Pass through graded lower concentration of alcohol and section/smear to bring in water.
- Nuclear stain: Alum haematoxylin for 10 min.
- Differentiates in acid alcohol.
- Wash in tap water.
- Stain in phloxine solution: 20 min.
- Wash in tap water.
- Keep in tartrazine: 5–10 min
- Dehydration by absolute alcohol
- Xylene
- Mount

Result

Viral inclusion: Bright red. Background: Yellow

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Part II

Basic Laboratory Techniques in Cytology Laboratory

13 Cytology Sample Procurement, Fixation and Processing

13.1 Introduction

The routine laboratory technique includes the following components:

- *Specimen collection*: Unlike histopathology, the specimen collection is variable in different body samples in cytology.
- *Fixation*: Different types of fixatives are used in various cytology samples.
- *Processing*: The processing of the cytology sample is widely different. It largely depends on the type of sample and available laboratory facilities.
- *Staining*: The common stains in cytology laboratory are Papanicolaou's and May Grunwald Giemsa (MGG) stain.

13.2 Sample Collection

The following broad types of samples are commonly received in cytology laboratory:

A. *Exfoliative cytology*
   - Cervical cytology: cervical smear, liquid-based cytology in vial
   - Respiratory samples: sputum, bronchial wash, bronchial brush, bronchoalveolar lavage, trans bronchial needle aspiration cytology, fine needle aspiration cytology (FNAC) under radiological guidance
   - Gastrointestinal tract: gastric brush, lavage, transendoscopic FNAC
   - Urine: voided urine, catheterized urine, ureteric urine
   - Effusion cytology: effusion fluid
   - Cerebrospinal fluid (CSF): clean vial
   - Vitreous fluid: clean vial
   - Joint fluid: in anticoagulant

B. *Fine needle aspiration cytology*: This is usually done directly by the cytologist.

13.2.1 Cervical Cytology [1, 2]

The cervical cytology sample is usually collected by the gynaecologist or the trained nurse. The collection of cervical cytology is important because the success of a cervical cancer screening program largely depends on adequate sampling.

Preparation of the patient

- Two weeks after the first day of the last menstrual period
- No sexual intercourse 2 days prior to sampling
- No use of vaginal cream, jellies or tampons

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_13

Collection devices

The ideal collection device should have the following properties:

- Able to collect materials from both ectocervical and endocervical regions
- Non-traumatic
- Non-sticky
- Cheaper
- Disposable

The following collection devices are available:

1. Wooden spatula (Fig. 13.1): cheaper, easy to handle, cells may be attached to wood
2. Plastic spatula: costlier than wood, non-sticking
3. Endocervical brush: easy to collect material, traumatic due to stiff bristle
4. Cervix brush (Fig. 13.2): (a) mainly used for liquid-based cytology (LBC) sample collection, (b) procure sample from both ecto- and endocervical material, (c) costlier than all the above devices

Collection Proper (Box 13.1):

- Keep the patient in dorsolithotomy position.
- Clean the vagina by wet swab.
- Do not use any lubricant jellies.
- Introduce a speculum to visualize the cervix.
- Inspect both the ectocervix and transformation zone; ectocervix is smooth pink and opaque, whereas the endocervix is dark pink.
- For the *conventional smear*
  - Broom or spatula is used.
  - Insert the spatula within the vagina so that the tip of the spatula fits with the contour of the cervix.

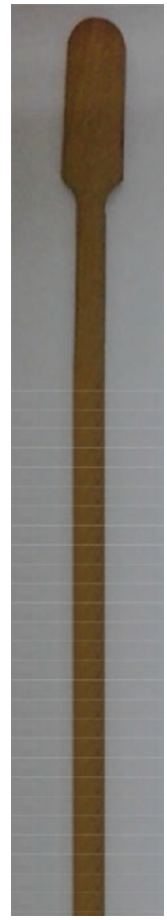

Fig. 13.1 Wooden spatula: this is a disposable spatula

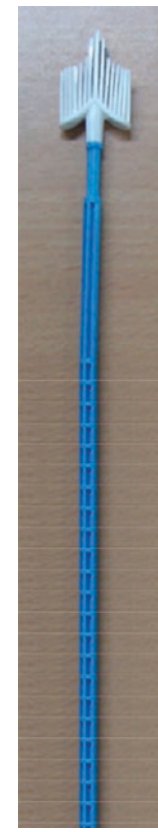

Fig. 13.2 Cervix brush: this is mainly used in liquid-based cytology

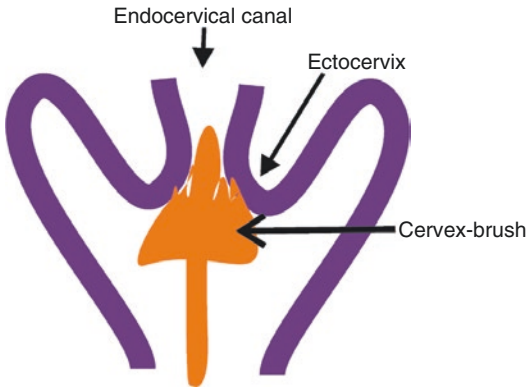

Fig. 13.3 Schematic diagram of cervical smear collection. The brush is introduced in the cervical canal the tip of the brush remains in the endocervical canal and the brooms are touched in the ectocervix. The brush is rotated and then withdrawn

  - Rotate the spatula in a complete round turn.
  - Withdraw it gently.
  - Spread the sample on the spatula on the glass slide.
  - Immediately fix the slide in 95% ethyl alcohol.
- For *LBC preparation*
  - Use cervix brush.
  - Introduce the central part of the broom into the endocervical canal so that the shorter bristles of the broom touch to the ectocervix (Fig. 13.3).
  - Rotate the broom four to five times in a clockwise direction.
  - Withdraw the broom and put it in the fixative solution as given by the company.
- Label the glass slide or the vial (for liquid-based cytology).

Vaginal smear: The cytology sample is collected from the lateral vaginal wall by using a spatula.

Endometrial aspiration smear: With the help of a sterile cannula and a syringe, the endometrial sample is aspirated.

Box 13.1: Cervical Smear Collection

Basic precautions

- Avoid vaginal cream, jellies or tampons 2 days prior to the test.
- Avoid collection during menstruation period.
- No sexual intercourse within 2 days.

Collection devices

For LBC: Cervix brush, LBC collection fluid in vial

For conventional:

- Endocervical broom stick, plastic spatula or wooden spatula
- Clean glass slides
- Permanent slide marker
- Fixative: 95% ethyl alcohol

Collection proper

Position of the patient: Dorsolithotomy

Preparation

- Open the vagina by speculum for proper visualization of the cervix.
- Clean the vagina by wet swab with water.
- Insert the cervix brush or plastic spatula within in the vagina.
- Rotate full circle.
- LBC: Rinse the brush in the fluid within the vial.
- Conventional:
  - Spread the material on the glass slide.
  - Immerse the smear immediately in 95% ethanol for fixation.

13.2.2 Respiratory Samples [3, 4]

The respiratory samples include sputum, bronchial brush, bronchial brush, bronchoalveolar lavage, transbronchial needle aspiration cytology and fine needle aspiration cytology (FNAC) under radiological guidance.

Sputum sample

- Collect the morning sputum in a wide-mouthed container.
- No fixative is needed.

Bronchial brush

- Visualized the lesion through the bronchoscope.
- Make the smear immediately on the slide.
- Prepare both alcohol-fixed and air-dried smears.

Bronchial wash

- Visualized the lesion through the bronchoscope.
- Introduce 5 ml normal saline through the bronchoscope.
- Aspirate the solution.
- Send the fluid immediately to laboratory.

Bronchoalveolar lavage (BAL)

- A fiberoptic bronchoscope is introduced under local anaesthesia in a selected subsegmental bronchus.
- Normal saline (20 ml) is flushed through bronchoscope.
- The lavage solution is recollected by gentle suction.
- The procedure is repeated four to five times.
- Overall 40–50 ml solution is procured.
- BAL fluid is sent to laboratory immediately.

Transbronchial needle aspiration

- The lesion is visualized by endoscopic ultrasound.
- A 22 gauze needle with an internal stylet is inserted through the endoscope to the mass under EUS guidance.
- The stylet is withdrawn, and aspiration is done by applying negative suction.
- The needle is withdrawn.
- The inside material is ejected by reintroducing the stylet within the needle.
- Multiple smears are made (both air-dried and alcohol-fixed).

Gastrointestinal Tract Gastric Brush, Lavage and Transendoscopic FNAC

Gastric brush:

- A flexible fiberoptic endoscopy is introduced in the stomach. The endoscope contains specific channels for biopsy forceps and brush.
- The brush is covered by a Teflon sheath to prevent the loss of cytology material.
- The mucosal lesion is visualized, and the brush is withdrawn from the outer Teflon sheath.
- The brush is gently rubbed on the lesion.
- Finally the brush is retracted into the Teflon sheath and is withdrawn from the endoscope.
- Multiple smears are made by rubbing the brush on the glass slide.

Gastric lavage:

- A flexible fiberoptic endoscopy is introduced in the stomach.
- Through the endoscope 100 ml normal saline is flushed in the lesion.
- By gentle suction the solution is withdrawn.
- It is repeated two to three times.
- Sample is sent to laboratory.

Endoscopic ultrasound-guided (EUS) FNAC:

- The lesion is visualized by endoscopic ultrasound.
- The FNAC needle is introduced through the channel of the fiberoptic endoscope.
- The needle is moved to and fro in the lesion maintaining negative suction.
- Finally, the needle is withdrawn, and the aspirated material is spread on the glass slide.

- Endoscopic FNAC is particularly helpful in submucosal tumours and is complementary to biopsy.

Urine Voided urine, catheterized urine and ureteric urine

Voided urine: Preferable for routine cytology

- Collect second voided urine.
- Collect urine in a clean container.
- No fixative.
- Send the sample in the laboratory immediately for processing.

Bladder wash:

- Introduce a catheter or cystoscope.
- Wash the bladder by 50–100 ml normal saline.
- Withdraw the solution.
- Sent the sample immediately to laboratory without any preservative.

Ureteric urine:

- The urine is collected from the each ureter by separate catheter.

*Urinary brush***:** With the help of a ureteric catheter, the lesion is brushed.

- The smears are made.

Effusion fluid sample:

- Collect fresh effusion fluid in a clean container.
- No fixative needed.
- To prevent coagulation 1:9 ratio of ammonium oxalate fluid can be used.
- In case of long delay in processing, an equal amount of 50% ethyl alcohol is mixed with the fluid.
- Do not allow the fluid to be frozen.

CSF and vitreous fluid

- To collect as fresh in a clean container
- Process immediately

13.3 Fixation

The fixatives in cytology should have same essential properties as described in histopathology sample in Chap. 1. The common fixatives in cytology include (Box 13.2):

- *Ethyl alcohol (95%)*: It is the most commonly used fixative. Ethanol causes dehydration of the cell and mild shrinkage.
- *Methanol (100%)*: Not cost-effective.
- *Denatured alcohol*: This is unsuitable for human consumption and so less chance of misuse.

Time of fixation

- At least 15–30 min.
- If necessary, one can keep the smear in fixative for long duration in a closed bottle or jar.

Box 13.2 Fixation

Ideal fixative

- Rapid action
- Prevention of cellular distortion
- Good nuclear details
- Facilitation of staining
- Inactivation of microbial organisms
- Fixing the cell on glass slide

Routine fixatives for Papanicolaou's and haematoxylin stains

- 95% ethyl alcohol
- 100% methanol
- Denatured alcohol
- ThinPrep system: Methanol-based preservative
- SurePath system: Ethanol-based preservative

Time of fixation: 15–30 min
Haemorrhagic fluid: Carnoy's fixative
Cell block: 10% neutral buffered formalin
Immunocytochemistry: 95% ethanol, cold acetone, etc. (cell block preferable)
Electron microscopy: Glutaraldehyde solution (2.5%)

Coating fixatives: Optimal distance of spray fixative is 12 inches away from the smear (Box 13.3). This prevents any distortion of morphology and damage of the smear.

Major advantages

- Easy to carry.
- Wax makes a protective covering over the smear.

Precautions

- The major ingredients of spray fixative are alcohol and wax. Therefore wax should be removed before staining.
- Maintenance of optimal distance.
- Bloody smear should not be fixed by spray fixative as this may cause RBC clumping.
- Fluid samples should not be fixed by spray.

Box 13.3: Spray Fixatives
Advantages

- Easy to carry

Functions

- Protection of smear by wax cover
- Cell fixation

Optimal distance of spray

- Overall 10 in away from the smear

Use

- Cervical smear

Not to use

- Smear of fluid specimen
- Smears with blood

Precaution: Wax should be removed by 95% alcohol before staining.

13.3.1 Special Fixatives

13.3.1.1 Haemorrhagic Fluid

Carnoy's Fixative Acetic acid in the solution lyses the RBCs. Cell morphology is well preserved in Carnoy's fixative. Main disadvantages of this fixative are (a) cell shrinkage and (b) nuclear overstaining.

Ingredients of Carnoy's fixative

- 95% ethanol (60 ml)
- Chloroform (30 ml)
- Glacial acetic acid (10 ml)

Use only fresh fixative and discard the remaining solution after use.

Fixatives for liquid-based preparation

ThinPrep system: Methanol-based preservative
SurePath: Ethanol-based preservative

Fixatives for cell block: Ten percent neutral buffered formalin is the best fixative for cell block materials.

Fixatives for immunocytochemistry: Cell block section should be used for immunocytochemistry.

- Routine fixative such as 95% ethanol can be used for immunocytochemistry.

Fixatives for electron microscopy: Glutaraldehyde solution (2.5%)

Preservation of sample prior to processing

- Process the specimen immediately.
- If the sample is not possible to process immediately, then it can be kept depending on:
  - Mucus content
  - Protein content
  - Sugar content
  - pH of the sample

Specimens with no sugar or protein, with extreme pH, should always be processed immediately (Fig. 13.4).

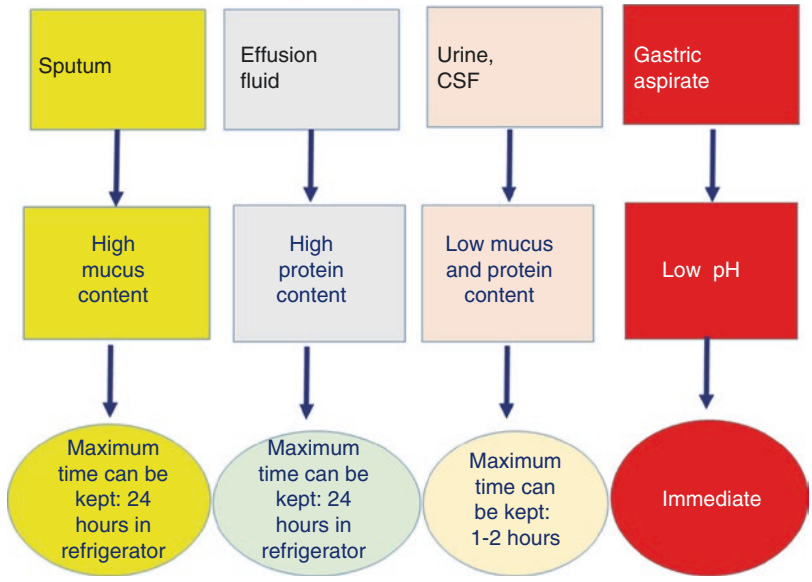

Fig. 13.4 Outline shows the maximum time duration between the collection of smear and processing

13.4 Processing of Laboratory Samples

Processing of a laboratory sample includes the following steps:

- Receiving
- Preparing smear
- Staining
- Mounting and final submission of the slide

13.4.1 Receiving the Sample

Requisition form: The sample should always be accompanied with a proper requisition form as mentioned in Fig. 13.5.

Requisition form

- Name Age Sex
- Unique identification of the patient
- Date of collection
- Site
- Procedure of collection
- Clinician's name and contact information
- Tests to be done
- Clinical history
  - Chief complaints
  - Physical findings
  - Radiological features
  - Important history: Surgery, chemotherapy, radiotherapy, exposure of chemicals etc.

Fig. 13.5 Requisition form of the cytology specimen

13.4.2 Glass Slides and Liquid Sample

The following precautions are essential regarding the receiving of glass slides:

- Slide should be received in a shock-resistant container.
- Smear should be properly fixed and labelled.
- Paper form should be in a separate bag, and slide should not be wrapped by requisition form.

Precautions for liquid samples:

- Container should be air tight.
- Properly labelled.
- Plastic container is preferable than glass container.

Unique identification number:

- Check the unique identification number of the sample and requisition form at the time of receiving and processing.

Laboratory bar code:

- Each sample should have a unique laboratory bar code number. This is separate from the unique identification number.
- Stick the bar code number on the container, smears and forms.

13.5 Processing

The commonly used processing techniques include:

(1) Direct smear
(2) Centrifuge
(3) Cytocentrifuge
(4) Liquid-based preparation
(5) Millipore technique
(6) Cell block

Figure 13.6 shows overview of processing of different samples.

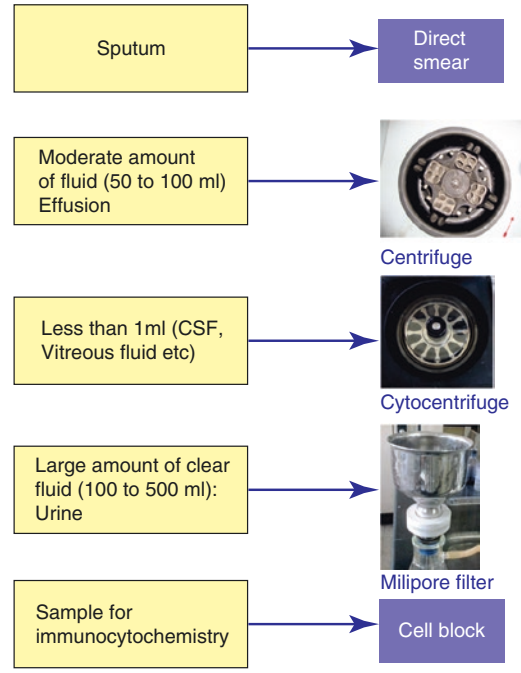

Fig. 13.6 Outline of the processing of different specimens. Sputum is traditionally processed by the direct smearing; moderate amount of fluid (up to 100 ml) is processed by centrifuge, whereas small volume (0.5 ml) of fluid is better processed by cytocentrifuge. Large amount of fluid is processed by Millipore filtration technique

13.5.1 Processing of Sputum (Fig. 13.7)

- Pour the sputum sample in a Petri dish kept on a black background.
- Carefully examined for any tissue fragments or grey-white substance or bloody material.
- Pick up the tissue fragments by a clean forceps.
- Prepare the smears on the clean glass slide.

13.5.2 Processing of Fluid: Urine, Body Fluids and Lavage

Centrifuge: The fluid of moderate amount (50–100 ml) should be processed by centrifugation, e.g. effusion fluid, turbid urine, etc. (Fig. 13.8).

- Put the fluid sample in clean air tight centrifuged tube.
- Rotate the tube at 1500 rounds per minute (RPM) for 10 min.
- Discard the supernatant liquid.
- Make multiple smears from the sediments.

Cytocentrifuge: Small amount of clean fluid such as 0.5–1 ml is processed by cytocentrifuge, e.g. CSF, ureteric urine, vitreous fluid, etc. (Fig. 13.9).

- Rotate the sample 1000 rounds per minute for 5 min.
- A thin layer of smear is formed on the glass slide.
- Fix the smears in 95% ethanol.

Basic Principle of Centrifuge Rapid circular movement of a particle around a central axis generates a centrifugal force that drives the par-

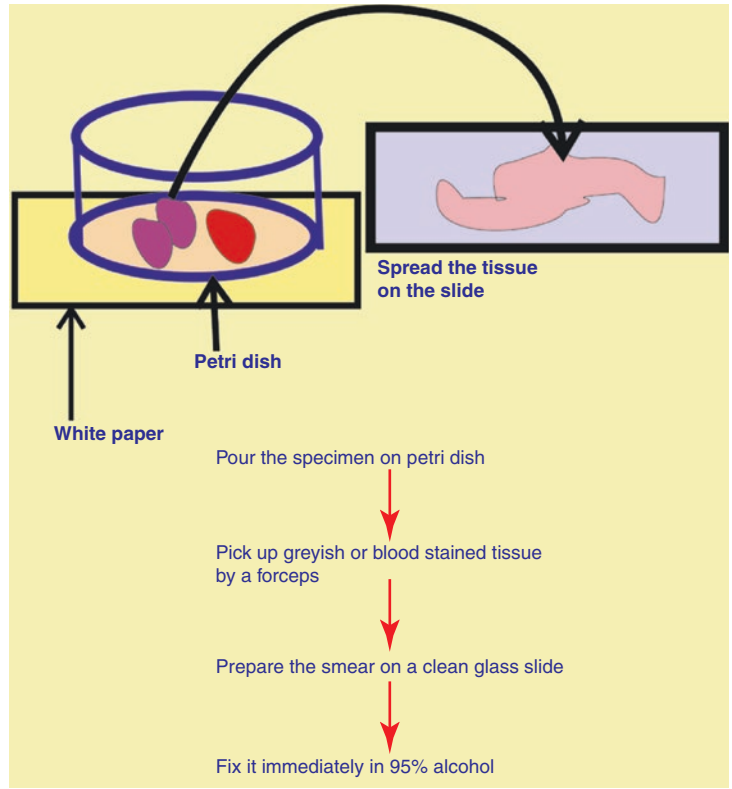

Fig. 13.7 Schematic diagram of processing of sputum

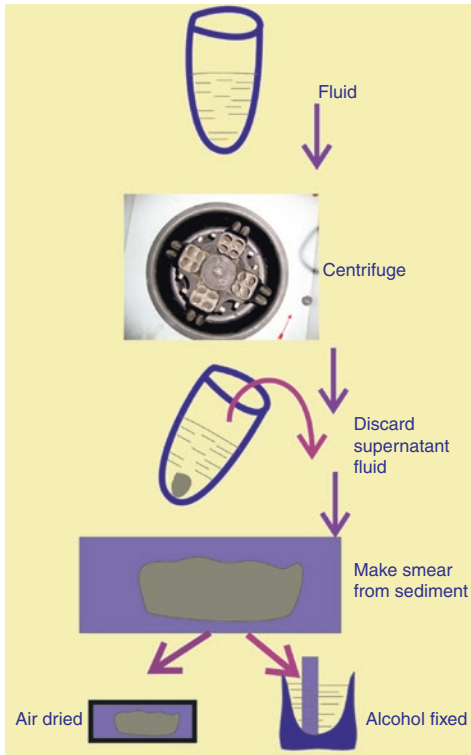

Fig. 13.8 Schematic diagram shows processing of fluid specimen

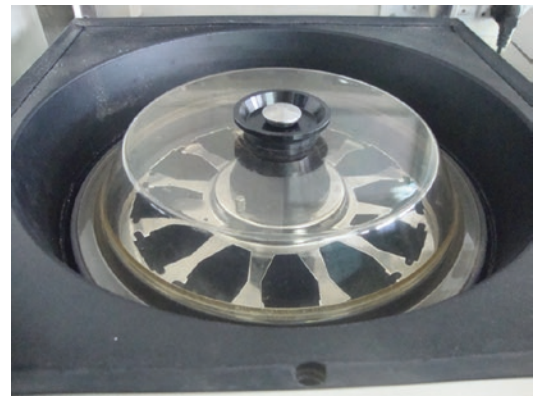

Fig. 13.9 Cytocentrifuge machine to process small quantity of sample

ticle away from the centre. This centrifugal force is counteracted by two other forces, (1) buoyant force that means floating capacity of the particle and (2) frictional force that develops due to friction of the particle and liquid media. We can express the forces by this equation:

$$\text{RCF} = 1.11 \times 10^{-5} \times (\text{round per minute})^2 \times r$$

$$\text{RCF} = \frac{r_{\omega}^{2}}{g}$$

Relative centrifugal force, RCF; rotational radius, r; angular velocity in radians per unit time, ω; gravitational acceleration, g

There are two types of commercially available cytocentrifuge:

1. The cytocentrifuge that removes the fluid during the time of sedimentation
2. The cytocentrifuge that retains the fluid

Cytocentrifugation may cause morphological distortion of the cells, and careful attention should be given in this aspect. The optimum rotational speed of the machine is the most important factor in this respect.

13.5.3 Millipore Filtration

The Millipore filtration technique is done for processing large quantity of clear urine sample.

- Put the moistened Millipore filter paper with normal saline on the sieve.
- Attach the filter cup.
- Put the sample in the filter cup.
- Put on the suction process.
- The negative pressure at 100 mm mercury is created, and the fluid is drained into the bottle leaving the cell on the filter paper (Fig. 13.10).
- Make multiple imprint smears on albumin-coated slides.
- Fix the slides immediately in 95% alcohol for 30 min.

13.5.4 Processing of Haemorrhagic Fluid

- *Carnoy's fixative*: This can be used for processing of haemorrhagic fluid.
- *Buffy coat preparation*:
  - Centrifuge the haemorrhagic sample in 2000 RPM for 10 min.
  - Discard the supernatant fluid.
  - Take the residual suspension in 2–3 cm long capillary tube.
  - Seal the one end of the tube.
  - Centrifuge the capillary tube.
  - Break the capillary tube in the buffy coat region.
  - Make fish tail preparation of smear.
- Commercially available lysing solution
  - Presently ThinPrep and SurePath companies supply the fixative solution that lyses the RBCs of the sample.

13.5.5 Cell Block [5]

The cell block technique is mainly used for:

1. Immunocytochemistry
2. Cytochemistry
3. Preservation of archival tissue for future use

The cell block is made by the following steps (Fig. 13.11):

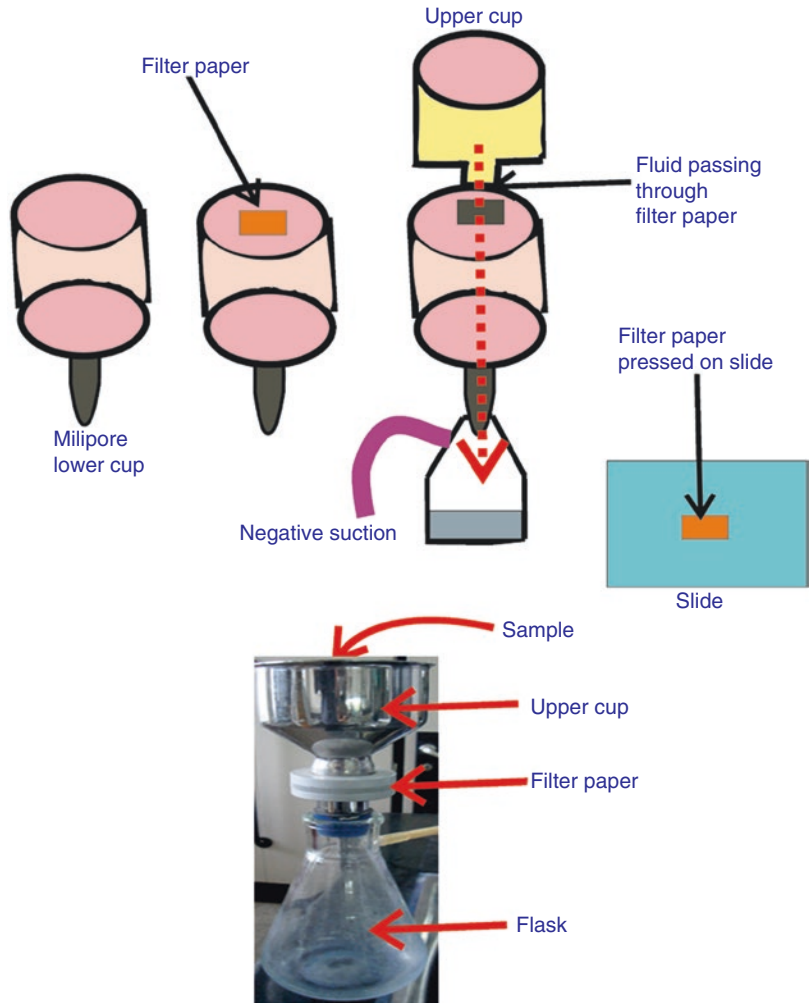

Fig. 13.10 Basic principles of the Millipore filtration technique. Here negative suction is used to draw the fluid from the upper container. The fluid passes through the filter paper, and the cell sticks on the upper surface of the filter paper

- Collect the specimen in 10% neutral buffered formalin.
- Keep it 4 h in formalin to fix the cells.
- Centrifuge the sample 1500 RPM for 10 min.
- Wash the sediment twice in PBS by centrifugation.
- Add 100 µl of plasma and 30 µl thrombin.
- Remove the clot and collect it on filter paper.
- Process the clot in the tissue processor as usual.

13.5.6 Compact Cell Block Technique

Young et al. [6] described compact cell block technique that is in a small area and completely free of RBCs.

Steps

1. Centrifuge the material in the fluid.
2. Add CytoRich solution (designed for haemolysis and gentle fixation of the cell) in 1:1 ratio to the sediment.
3. Keep it for 2 min.
4. Add four drops of plasma and three drops of thrombin (5000 IU/ml).
5. Agitate the mixture gently to make a cell clot.
6. Transfer the clot into the lens paper.
7. Wrap the compact clot and then fix into formalin.
8. Process the clot in the tissue processor.

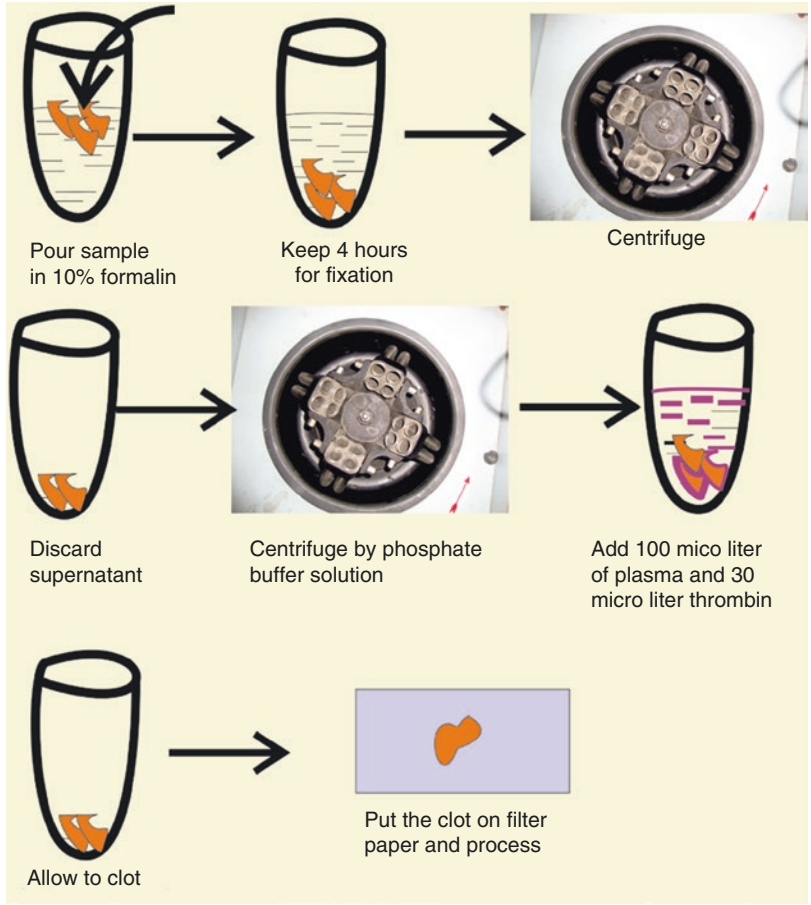

Fig. 13.11 Basic steps of cell block preparation are highlighted in this picture

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Routine Staining in Cytology Laboratory

14

The most commonly used two routinely available stains in the cytology laboratory are

1. Papanicolaou's stain
2. May Grunwald Giemsa stain

14.1 Papanicolaou's Stain [1]

Dr. George Papanicolaou, the father of cytopathology, first introduced this stain. Papanicolaou's stain (PAP stain) is the most important stain in cytology and is used in all cervical smear and non-gynaecologic exfoliative smears. This stain has the following excellent properties:

- Cytoplasmic differentiation: It helps in the assessment of cellular differentiation.
- Nuclear details seen.
- Transparent stain.
- Demonstrates intracytoplasmic keratin.

Progressive method: As mentioned before, in case of progressive stain, the nuclei are optimally stained, and the cytoplasm does not take the dye.

Regressive method: In regressive staining, the nuclei are intentionally overstained by haematoxylin dye. Subsequently the excess stain is removed by acid alcohol.

14.1.1 Dyes Used in Papanicolaou's Staining

Haematoxylin: Harris haematoxylin for nuclear stain.

Orange G: OG-6 for cytoplasmic counter stain. It also stains keratin component of the cytoplasm.

Eosin Azure (EA): It is a polychrome stain and consists of three dyes: Eosin Y, light green SF yellowish and Bismarck brown Y.

14.1.2 Principle of Basic Steps (Fig. 14.1)

1. *Rehydration of the smear*: With the help of subsequent dip in the graded concentration of alcohol.
2. *Nuclear staining by haematoxylin*: Harris haematoxylin is a good rapid nuclear stain. Subsequent differentiation is done to remove excess haematoxylin by acid alcohol.
3. *Bluing*: This is done by treating the smear with running tap water; alternatively weak alkaline solution can be used.
4. *Cytoplasmic staining by Orange G (OG)*: As OG is alcohol-soluble dye, so the smear is again brought into alcohol and stained with OG.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_14

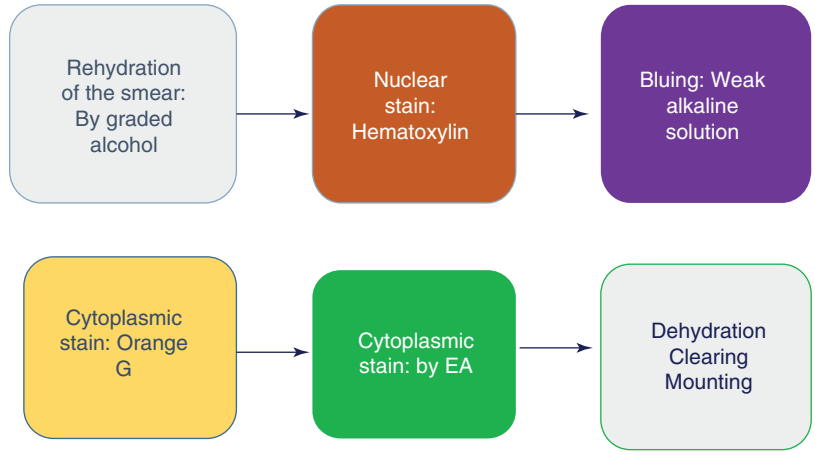

Fig. 14.1 Basic principles of Papanicolaou's stain are highlighted in this diagram

5. *Cytoplasmic staining by EA*: The cell cytoplasm is stained as blue-green colour by EA.
6. *Dehydration*: It is done by absolute alcohol.
7. *Clearing*: Xylene.
8. *Mounting*: By DPX mounting medium.

14.1.3 Papanicolaou's Staining Steps

1. 70% ethanol: 1 min
2. 50% ethanol: 1 min
3. Distilled water: 5 dips
4. Harris haematoxylin: three and half minutes
5. Distilled water: 5 dips
6. 0.25% aqueous solution of hydrochloric acid: few dips
7. Water: 1 min
8. Lithium carbonate: one and half minute
9. Water: few dips
10. 70% ethanol: 2 min
11. 90% ethanol: 2 min
12. Orange G: few dips
13. 95% ethanol: 2 min
14. EA modified: 2 min
15. Absolute ethyl alcohol: 2 min
16. Absolute ethyl alcohol: 2 min
17. Absolute ethyl alcohol and: 2 min
18. Xylene: 5 min
19. Xylene: till clear
20. Mounting in DPX

14.1.3.1 *Result* (Figs. 14.2, 14.3 and 14.4)

Nuclei: Dark blue

Cytoplasm: Blue green

Keratin: Orange

Figures 14.2, 14.3 and 14.4 describe the normal cervical cytology smear, high-grade squamous intraepithelial lesion and squamous cell carcinoma, respectively, of the routine cervical smear. These are the routine cervical smears of the liquid-based cytology preparation in our laboratory in Post Graduate Institute of Medical Education and Research, Chandigarh, India.

Haematoxylin Solution for Papanicolaou's Stain

Harris haematoxylin	5 gm
Distilled water	1000 ml
Alum	100 gm
Absolute alcohol:	50 ml
Mercuric oxide	2.5 gm

- Dissolve haematoxylin 5 gm in 50 ml alcohol.
- Mix 100 gm alum in water and boil it to dissolve.
- Now mix haematoxylin and alum in water and boil.
- Take out the flask from heat.
- Put 2.5 gm mercuric oxide in the solution.

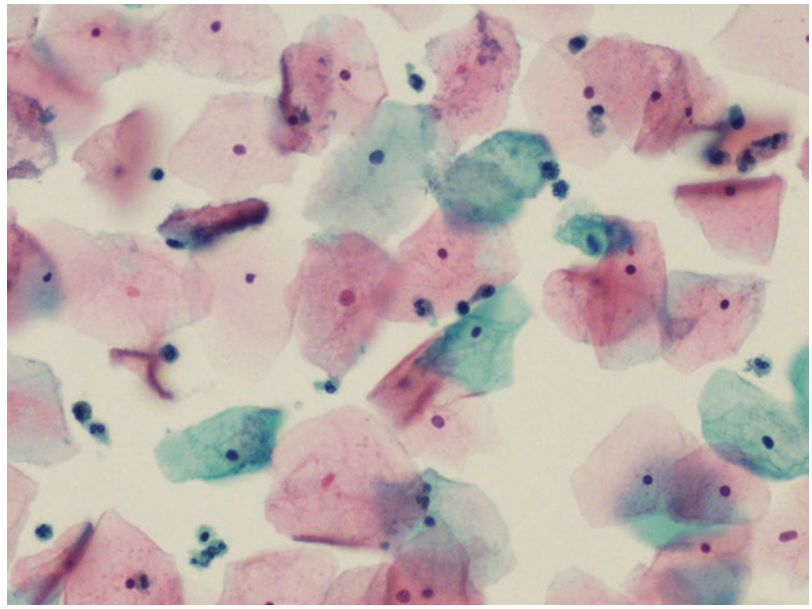

Fig. 14.2 Papanicolaou's stain of the normal cervical smear (SurePath preparation of Papanicolaou's stain ×240)

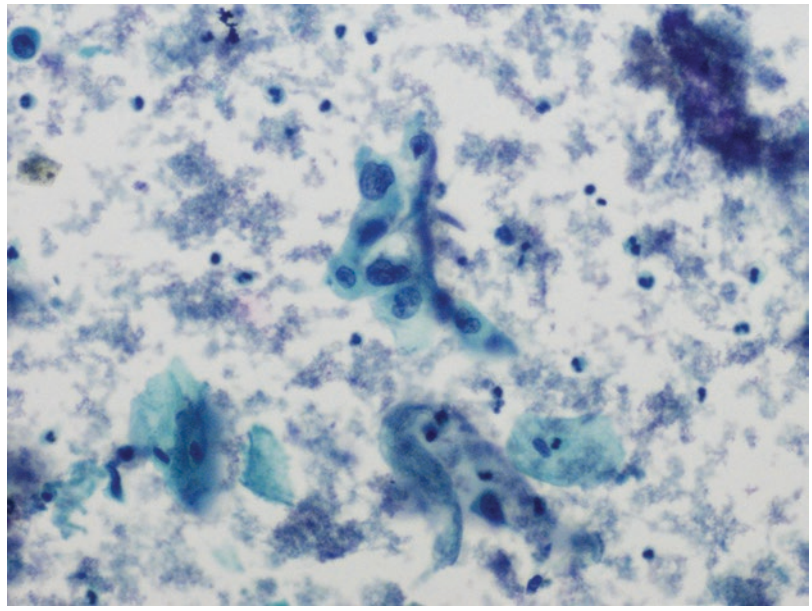

Fig. 14.3 Papanicolaou's stain of the cervical smear in a case of high-grade squamous intraepithelial lesion (SurePath preparation of Papanicolaou's stain ×440)

- Mix thoroughly by stirring till the colour becomes dark purple.
- Cool the flask.
- Filter.
- Store in dark place.

EA solution

Eosin	2 gm
Light green	0.2 gm
Water	240 ml
Alcohol	250 ml
Phosphotungstic acid	1 gm
Glacial acetic acid	10 m

Orange G solution

Orange G (10% aqueous)	50 ml
Ethyl alcohol (95%)	950 ml
Phosphotungstic acid	0.15 gm

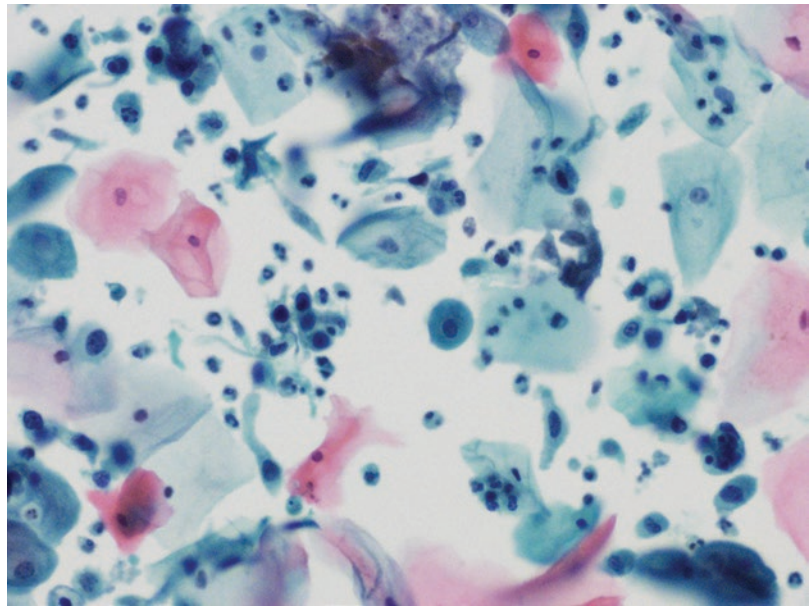

Fig. 14.4 Papanicolaou's stain of the cervical smear in a case of squamous cell carcinoma of the cervix (SurePath preparation of Papanicolaou's stain ×440)

14.1.4 Bluing Solution

14.1.4.1 Lithium Carbonate Solution

Stock solution

Lithium carbonate ($LiCO_3$)	1.5 g
Water	100 ml

Working solution

Water	1000 ml
Stock solution of lithium carbonate	30 drops

14.2 Precautions to Be Taken in Papanicolaou's Staining

The following precautions should be taken in Papanicolaou's staining:

Staining Solutions

- The container should be covered; otherwise, the evaporation of water or alcohol may change the concentration of the dye.
- The containers should be washed regularly.
- Haematoxylin solution: It is a stable solution. However the addition of small amount of fresh dye solution increases staining quality.
- Orange G: It is less stable than haematoxylin, and the strength reduces quickly within days. Replace OG whenever the cytoplasmic colour appears less crispy.
- Xylene: It may be contaminated with the staining dye. Change immediately if it is tinted.
- Alcohol: Check the concentration of the alcohol on regular basis. Discard the first container of alcohol, and replace it with the second and third.
- The stain solution should be free of stain deposits and should be filtered daily.

Coverslip

- Smears should not dry before placing the coverslip.
- No air bubbles should be present between the coverslip and smear.

Staining Proper

- Gentle and slow agitation of the slides within the staining solution helps to prevent staining deposit and also gives better staining.
- Rapid agitation may cause dislodging the cells.
- Stain rack should not hit the bottom of the container.

Table 14.1 Troubleshooting areas of Papanicolaou's stain

Problems	Possible causes	Remedies
Too dark nuclear stain	• If the smear is kept in Harris haematoxylin for a long time	• Keep less time in Harris haematoxylin
	• Too less hydrochloric acid concentration	• Maintain the concentration of acid
	• Too less number of dips in hydrochloric acid	• Differentiates properly
	• Too much bloody smear or high protein content of the smear	• Try to take better smear
Too pale nuclear stain	• If haematoxylin is diluted	• Maintain the proper dilution of haematoxylin or change the solution
	• Too less bluing	• Give time for bluing
	• Too much concentration of hydrochloric acid	• Maintain the concentration of acid
	• Too much dip in hydrochloric acid	• Control this step and give less dips in acid
	• Air-dried smear prior to fixation	• Try to fix the smear properly in the future
Improper cytoplasmic stain	• Smear is air-dried prior to fixation	• Try to fix the smear properly in the future
	• If the slides remain in alcohol for too long time, then the cytoplasmic stain may be pale	• Maintain the time of fixation in alcohol
	• If the pH of EA is not optimum	• Maintain pH of EA solution
	• If the slide remains in haematoxylin for too long time	• Control the staining time in haematoxylin

Table 14.1 highlights the troubleshooting areas of the Papanicolaou's stain.

Destaining and Restaining of the Smear

- Dip the smear in xylene until the coverslip drops.
- Keep the smear in acid alcohol for 20 min (80 ml of 95% ethanol and 20 ml of 0.5% hydrochloric acid).
- Wash the smear with running water.
- Restain.

14.3 May Grunwald Giemsa Stain

May Grunwald Giemsa (MGG) is a Romanowski's stain and is routinely used in many laboratories. This stain provides excellent cytoplasmic detail character (Fig. 14.5). This is a metachromatic stain. MGG is a convenient stain for FNAC smear. Table 14.2 compares the relative advantages and disadvantages of MGG stain over Papanicolaou's stain.

Steps

1. May Grunwald solution: 5 min
2. Running water: 1 min
3. Giemsa stain: 15 min
4. Running water: 1 min
5. Air-drying

Clearing and mounting have been discussed in Chap. 8.

Storage of Slides

- All the positive slides should be stored for indefinite period.
- The negative slide should be stored for a minimum of 5 years.

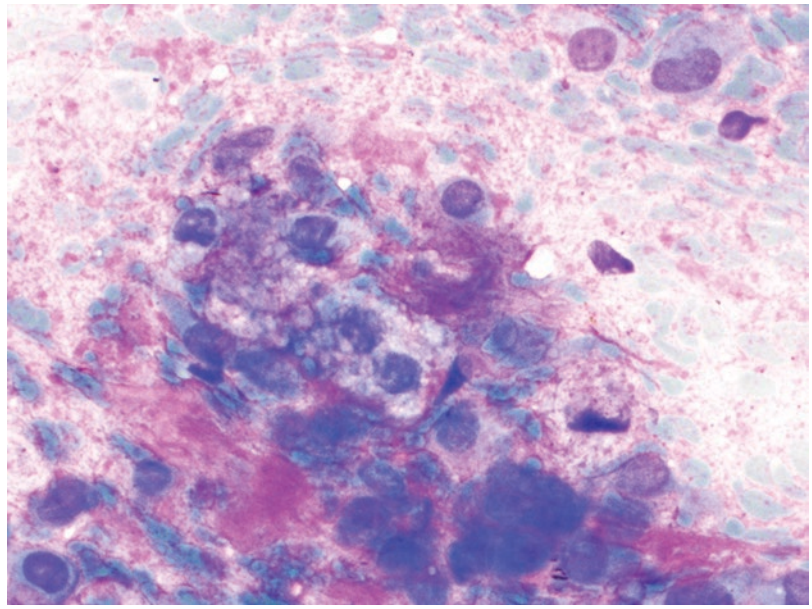

Fig. 14.5 May Grunwald Giemsa stained smear in a case of pleomorphic adenoma. Note the metachromatic staining of the chondromyxoid substances as deep magenta-coloured material (May Grunwald Giemsa stain ×440)

Table 14.2 Comparison of Papanicolaou's stain and May Grunwald Giemsa stain

Features	PAP stain	MGG stain
Nuclear detail	Excellent and very good stain for chromatin stain	The chromatin pattern cannot be studied
Keratin demonstration	Orange G stains keratin as bright orange colour	Cannot be demonstrated
Metachromasia	Not a metachromatic stain	Metachromatic stain
Transparency	Transparent stain	Not a transparent stain
Background mucin or necrosis	Not good	Good for demonstration of extracellular substance

MGG May Grunwald Giemsa, *PAP stain* Papanicolaou's stain

Reference

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15 Basic Technique of Fine Needle Aspiration Cytology

15.1 Introduction

Fine needle aspiration cytology (FNAC) is a major area of cytology. With the help of FNAC, cytologists can procure sample from any part of the body. It is an easy and safe technique and can be done as routine office procedure [1]. FNAC does not require any anaesthesia or special precautions. This is an economical, cost-effective and highly specific technique. FNAC is comparable or even better than the frozen section in certain situations such as thyroid or breast lesions. Presently many ancillary techniques can be done on FNAC material, and therefore it is possible to provide exact diagnosis with the help of this technique (Box 15.1).

Box 15.1 FNAC: Advantages

- Simple
- Easy to do
- Rapid
- Cheap
- Multiple area of sampling
- High sensitivity and specificity
- Bypass tissue biopsy
- Ancillary techniques possible on FNAC sample

Table 15.1 Tissue biopsy versus fine needle aspiration cytology

Features	FNAC	Surgical biopsy
Tissue architecture	Lost	Preserved
Lymphatic and capsular invasion	Impossible to assess	Possible to assess
In situ carcinoma	Difficult to diagnose	Possible to diagnose
Individual cytomorphology	Excellent	Not so good as that of FNAC material
Immunocytochemistry and other tests	Possible	Possible

FNAC fine needle aspiration cytology

Tissue Biopsy Versus FNAC (Table 15.1) The loss of architecture of the tissue is the major limitation of this technique. In situ versus invasive lesions are often difficult to predict on FNAC material. Moreover capsular and lymphatic invasion cannot be assessed on FNAC sample. In fact, special skill is needed to report FNAC material as it is quite different from tissue biopsy [2].

Exfoliative Cytology Versus FNAC FNAC sample is also different than the exfoliative cytology. FNAC sample shows limited tissue architectural pattern along with well-preserved cell morphology. The overall cellular characteristics are important in FNAC sample compared to exfoliative cytology (Fig. 15.1).

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_15

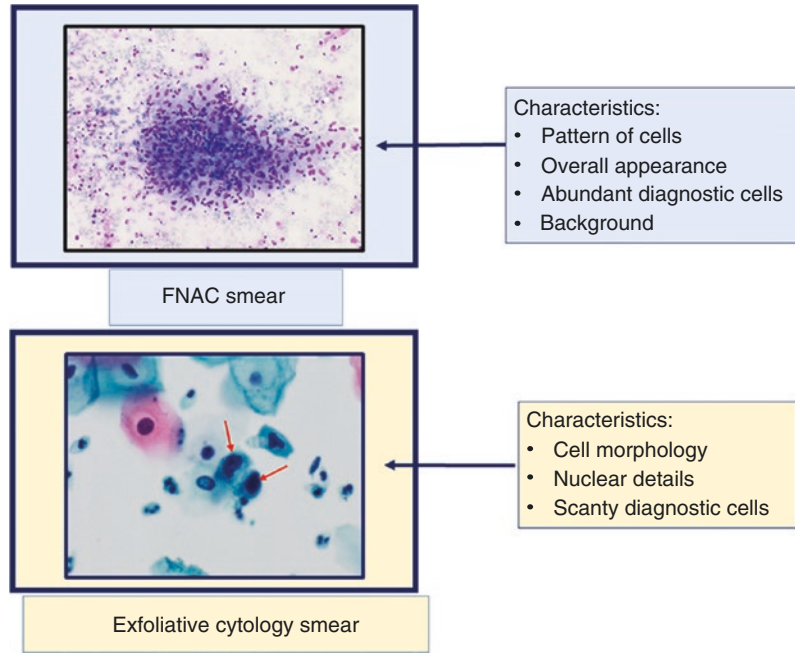

Fig. 15.1 Comparison of exfoliative cytology and FNAC

Complications FNAC of superficial masses does not have any significant complications except minor haematoma. FNAC of deep-seated masses are usually safe. Rarely following complications may occur:

- Surgical emphysema in lung FNAC
- Rupture of aneurysmal vessel
- Anaphylaxis in case of hydatid cyst
- Biliary peritonitis and bowel perforation
- Needle tract seedling of cancer cells (if thick bore needle is used) [3]

Contraindications FNAC technique does not have any contraindications. It should not be done in vascular organ in case of coagulation disorders such as haemophilia or thrombocytopenia.

15.2 Technique Proper

Equipment (Fig. 15.2)

- *Pistol handle to hold the syringe*: The pistol has the provision to attach syringe within it. The pistol handle helps to hold the syringe properly also to apply negative suction at the time of FNAC. The one hand of the operator remains free to hold the swelling.
- *Syringe*: Disposable 10 or 20 ml plastic syringe.
- *Needles*: Ordinary disposable hypodermic needle of 22–25 gauge is needed.
  - Large bore needle: For hard and fibrotic lesion or cyst with viscous material.
  - Thin bore needle: For small lymph node or vascular organ-like thyroid.
- *Clean glass slides*: Labelled clean glass slides.
- *Spirit swabs*: Clean sterile sprit swabs to clean the area of aspiration.

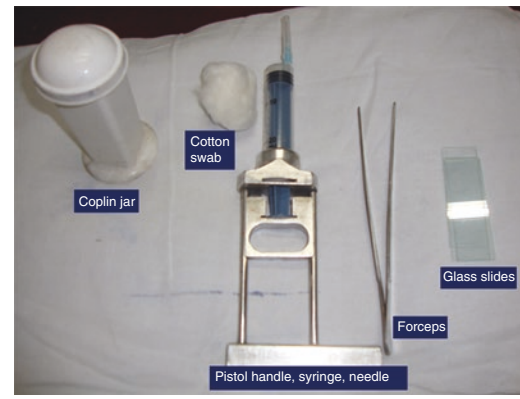

Fig. 15.2 Essential equipment to do FNAC

- *Suitable fixatives*: 95% ethanol for fixation of slide.
- *Additional*
  - Few capped vials containing 10% formalin solution for cell blocks.
  - Few capped vials containing balanced salt solution for flow cytometry.
  - Clean sterile vial for culture (bacterial, fungal, etc.).
  - Vials for PCR and other molecular techniques.

15.3 Fine Needle Aspiration Procedure (Box 15.2)

Clinical History The following information are mandatory before FNAC:

- Exact site of swelling
- Size of the lesion
- Chief complaints with duration
- History of previous FNAC
- Any bleeding disorder

Preparation of the Patient The following measures may help during preparation of the patient:

- Explain the whole procedure in brief.
- Take proper consent from the patient particularly for FNAC of deep-seated lesions and orbit.
- Talk with the patient and give assurance to make him/her relax.
- Clean the area of the site of FNAC with a spirit swab.

Aspiration (Fig. 15.3)

- The cytopathologist should personally perform FNAC [4].

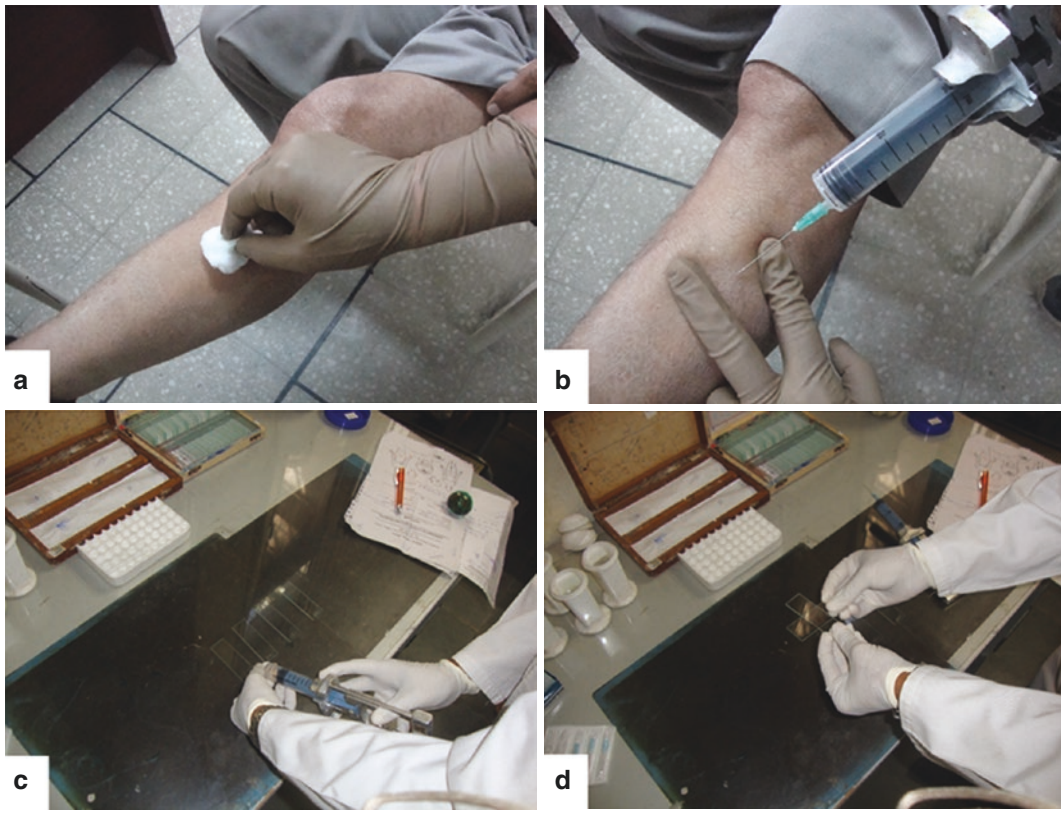

Fig. 15.3 FNAC technique is demonstrated on dummy patient: (**a**) The area of FNAC is cleaned. (**b**) The needle attached with the syringe is introduced into the swelling and negative suction is given along with to and fro movement. (**c**) The material is expelled on the glass slide. (**d**) The smear is made

- Take the pistol handle with attached plastic syringe and needle.
- Immobilize the swelling by two fingers of one of your free hands.
- Gently introduce the needle and move the needle to and fro in the mass.
- Apply negative suction by withdrawing the plunger.
- Lastly, release of the plunger to stop negative suction.
- Withdraw the needle.
- Apply firm pressure in the site of FNAC to stop any bleeding.
- Retract the plunger to get enough air within the syringe.
- Reattach the needle.
- Eject the material on the slide.

Smear Preparation

- Push the material on a clean glass slide.
- Material should be a few cm away from the end of the slide.
- Needle should be parallel to the slide and little bended.
- Make smear by gently pressing a clean glass slide over the lower one to spread the material.
- Make 3/4 smears.
- Keep both air-dried and alcohol-fixed smears.

15.4 Fine Needle Sampling

Fine needle sampling (FNS) is done without using any syringe. No artificial negative suction is applied, and the material is aspirated with the help of negative capillary pressure of the atmosphere [5].

Steps

- Clean the area by sprit swab.
- Press the swelling in between the two fingers to make it prominent.
- Introduce a thin bore needle within the swelling.
- Move the needle to and fro in the same direction and also slowly in different directions.
- The material comes to the hub of the needle by capillary pressure of the atmosphere.
- Gently withdraw the needle.
- Fill the syringe with air, and attach the needle with the syringe.
- Expel the aspirated material on the glass slide.
- Make the smears.

Advantages The major advantages of FNS are:

- To get material without any admixture of blood.
- FNS is helpful in vascular organ-like thyroid.
- Small swellings are often difficult to fix and can be done by FNS.
- Easy to manipulate the needle as it is light and without any attachment of syringe.

Box 15.2: Fine Needle Aspiration Procedure

- Clinical information:
  - Location
  - Size of the swelling
  - Duration of the lesion
  - Major complaints
  - Any history of coagulation disorder
- Preparation of the patient:
  - Discuss the technique.
  - Take written consent.
- Aspiration:
  - Clean the area of aspiration by spirit.
  - Hold the swelling in between the two fingers to make it immobilized.
  - Insert the needle within the mass.
  - Make to and fro movement of the needle.
  - Apply negative suction.
  - Stop the suction.
  - Withdraw the needle.
  - Detach the needle.
  - Fill the syringe with air.
  - Reattach the needle.
  - Eject the material on the glass slide.
  - Make adequate number of smears.
  - Apply firm pressure on the site of FNAC with the help of cotton swab.

Box 15.3: Fine Needle Sampling Technique

Indications

- All vascular tissue particularly thyroid
- Small mobile swelling that are difficult to fix

Advantages

- Completely painless
- Absence of blood
- Rich in material

Limitations

- Chance of spillage in cyst
- Less volume
- Unsuitable for bony and fibrotic lesions

Limitations

- The overall material is less in volume and therefore difficult to get multiple smears.
- There is a chance of spillage in cystic lesion.
- FNS is not suitable for bony and fibrotic lesion.

15.5 FNAC of Deep-Seated Lesions

The basic technique of FNAC is similar in both superficial lesion and deep-seated lesions. However, appropriate radiological guidance is needed to localize the deep-seated lesion. The common types of guidance of deep-seated FNAC include:

- Ultrasonography (USG)-guided FNAC
- Computerized tomography (CT)-guided FNAC
- Endoscopic ultrasound-guided FNAC (EUS-FNAC)
- Mammographic-guided FNAC
- Fluoroscopy-guided FNAC
- Magnetic resonance image (MRI) guidance

Advantages of Guided FNAC The major advantages of guided FNAC are:

- Easy to sample from the area of particular interest (representative part of tissue)
- Avoids any injury to major structures
- Avoids aspiration from the necrotic tissue or cystic area
- Possible to aspirate from the tiniest lesion
- Helps to aspirate from the deep-seated non-palpable lesion

Major indications of deep-seated guided FNAC:

- Diagnosis of the primary lesion
- Assessment of staging of the malignant lesion
- To collect material for ancillary studies such as flow cytometry, microbial culture, etc.

Ideal Radiological Imaging Modality The ideal radiological imaging modality should have following qualities:

- Free from any radiation hazard
- High-resolution image generation
- Real-time visualization of the needle
- To localize the smallest lesion

Essential Information for the Guided FNAC The following information are essential before performing guided FNAC:

- Clinical history
- Simple X-ray, USG or CT scan picture
- Size of the lesion
- Routine blood test
- Coagulation parameters

Fluoroscopy-Guided FNAC This is particularly helpful in case of FNAC of cortical bony lesion.

Advantage: Easiest method to do

Disadvantage: High radiation exposure so adequate shield is necessary.

15.5.1 USG-Guided FNAC

This is the most popular and simple technique without any radiation hazards. USG machine is portable and cheap. With the help of USG, the radiologist can monitor the passage of the needle by real-time monitoring at the time of FNAC. Therefore it helps to insert the needle in the exact position within the lesion. USG-guided FNAC can be done from any part of the body. However this is widely used in intra-abdominal organs, small thyroid lesions and breast lesions (Fig. 15.4).

Principle In case of USG-guided FNAC, high-frequency sound wave is used. The reflection of the wave from tissue interface is recorded, and the image is constructed.

Advantages The major advantages of USG-guided FNAC are:

- Economic.
- Portable.
- Rapid.
- Easy to do.
- Real time.
- No radiation exposure.
- Angular approach can be done.

Disadvantages

- Significant obscuration due to air or bone may affect the image.
- Not very high resolution.
- Completely operator dependent.

Procedure

- Identify the lesion by USG.
- Introduce the needle with stylet.
- Withdraw the stylet.
- Apply suction with the help of syringe.
- Withdraw the needle and stylet.
- Spread the material over the slide.

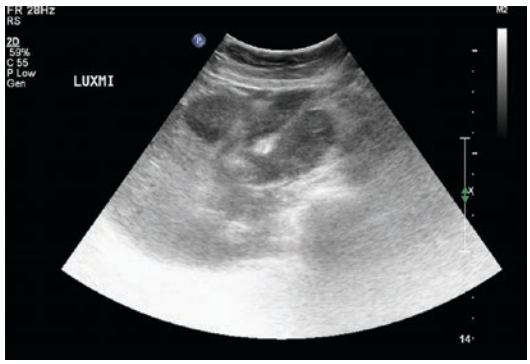

Fig. 15.4 USG-guided FNAC of pancreatic tumour (Courtesy by Prof Anupam Lal, Radiology Department, PGIMER, Chandigarh)

15.5.2 CT-Guided FNAC

Computed tomographic scan (CT scan) is also the popular and useful for deep-seated FNAC.

Principle CT uses X-ray beam to create multiple tomographic or cross-sectional images of the body depending on the difference of physical density of the tissue. The computer accumulates all the data and produces multiple images in different planes. Multiple images of few mm thickness are generated. Therefore small and inaccessible lesion can also be reached by CT scan.

Advantages

- High resolution.
- Exact localization of the needle possible.
- Operator independent.
- Deep lesion near vital structure needs CT guidance.

Disadvantages

- Costly
- Time taken procedure
- Good radiation exposure

Application Small lesions situated near vital structures such as mediastinal lesions or small lung lesions.

15.5.3 Endoscopic Ultrasound-Guided FNAC (EUS-FNAC)

Here FNAC is done through endoscope under the guidance of USG [6]. EUS-FNAC can be done

from mediastinal, lung, oesophageal, biliary tree and pancreatic lesions.

Steps
- Localize the lesion by endoscopic ultrasound.
- Insert the needle with the stylet through the endoscope to the mass under EUS guidance.
- Withdraw the stylet and aspirate with the help of 20 cm^3 syringe by applying negative suction.
- Do FNAC from multiple sites.
- Finally withdraw the needle, and expel the material on the slide by reintroducing the stylet within the needle.

Others Magnetic resonance imaging (MRI) uses radiofrequency energy and is free from any radiation hazards. MRI-guided FNAC is still not popular because of longer time to do, high cost and complicated procedure. In addition, mammographic-guided FNAC or core needle biopsy is also popular nowadays. Core needle biopsy has the added advantage to detect the in situ carcinoma of the breast, and therefore it has almost replaced the FNAC of breast [7].

15.5.4 Complications of Guided FNAC

There is no significant complication in the guided FNAC. However, occasionally, there may be bleeding, medical pneumothorax or infection.

15.6 Transrectal FNAC of the Prostate

Transrectal FNAC of the prostate is easy to do, and it can be done in outdoor basis. It samples from the different areas of the prostate in a single sitting.

Steps (Figs. 15.5 and 15.6)
- Keep the patient in his left lateral position with the lower leg extended.
- Palpate the prostate first.

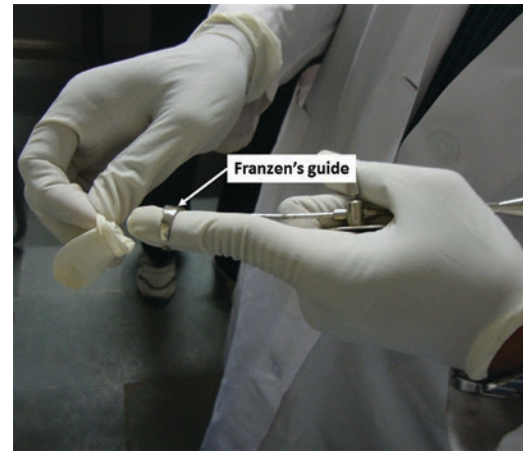

Fig. 15.5 Franzen's guide is fixed with the left index finger

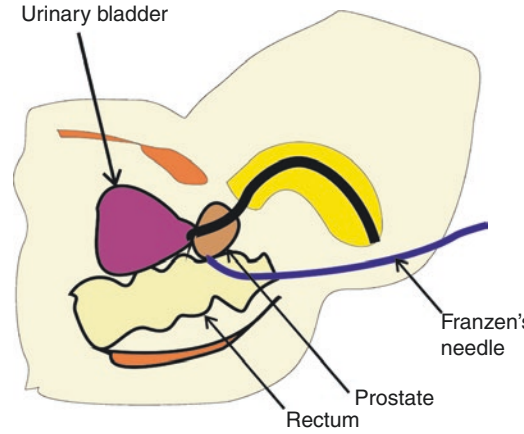

Fig. 15.6 Schematic diagram showing transrectal fine needle aspiration cytology with the help of Franzen's needle

- Fix Franzen's guide in the left index finger with the help of finger stall.
- Now introduce Franzen's guide gently through the rectum to the area of the lesion of prostate.
- Introduce Franzen's needle with attached syringe within the guide.
- Do FNAC with the help of Franzen's needle and syringe.
- Take out the needle and syringe.
- Expel the aspirated material on the slide.
- Make multiple smears.

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Part III

Advanced Techniques in Histology and Cytology Laboratories

Immunocytochemistry in Histology and Cytology

16

16.1 Introduction

Immunohistochemistry (IHC)/immunocytochemistry (ICC) is the technique to visualize recognition of antigen present in the tissue with the help of corresponding antibody. Coons et al. first time applied immunofluorescence technique on the frozen section by using fluorescence labelled antibodies [1]. The antibody conjugated with enzyme acid phosphatase and horseradish peroxidase was used first time by Nakane and Pierce in 1967 [2]. IHC technique was successfully introduced in routine formalin-fixed paraffin-embedded (FFPE) section by Taylor and Burns in 1974 [3]. Subsequently the development of monoclonal antibody introduced a new era in the immunohistochemistry [4]. However, it took another 10–15 years to have regular routine use of IHC in pathology diagnostic laboratory. Presently IHC is an essential technique in every pathology laboratory.

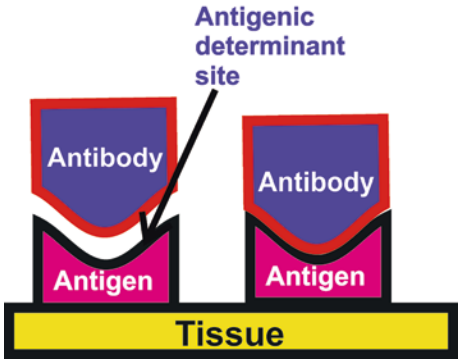

Fig. 16.1 Antigenic determinant site provokes antibody formation

16.1.1 Basic Principles

The basic principle of immunocytochemistry is to demonstrate the specific antigen in the cell by applying the corresponding antibody to have antigen-antibody reaction. The antigen contains an epitope or antigenic determinant site that evokes specific immunologic response to develop antibody. The antigen epitope site and antibody-binding site have complementary geometrical and chemical features (Fig. 16.1). This is responsible for the antigen-antibody reaction. This antigen-antibody reaction is further visualized by attaching certain label to the primary or secondary antibody.

16.1.2 Basic Immunology

Antigen Any substance that is capable of producing an immunogenic response is called as antigen. There are specific set of chemical components that evoke immunogenic response of the antigen which is known as epitope or antigenic determinant site.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_16

Antibody (Immunoglobulin) Antibody is also known as immunoglobulin. The antibody is produced by plasma cells in response to antigenic stimulation. Immunoglobulin has specific affinity against the epitope of the antigen. Each immunoglobulin is composed of a pair of light chains and a pair of heavy chains polypeptides. The light chains of immunoglobulin are of two type κ (kappa) and λ (lambda). There are five types of heavy chain, α (alpha), γ (gamma), δ (delta), ε (epsilon) and μ (mu), and depending on the nature of heavy chain, immunoglobulins are labelled as IgA, IgG, IgD, IgE and IgM, respectively. The antibody is a Y-shaped structure. The two tips of the Y are known as antigen-binding site of the immunoglobulin (Fig. 16.2). The base of each arm of Y is the hinge region that is flexible region of the immunoglobulin.

Hybridoma Technique In this technique abundant unlimited amount of pure homogenous immunoglobulin is produced. The antibody producing B lymphocyte is fused with a malignant immortal plasma cell. The resultant hybrid cell acquires the capability of unlimited proliferation and production of specific antibody. The resultant antibody in this condition is known as "monoclonal antibody". They are named as "monoclonal" because they are produced from a single clone of cell. Each hybridoma cell produces only a specific antibody for a specific antigen.

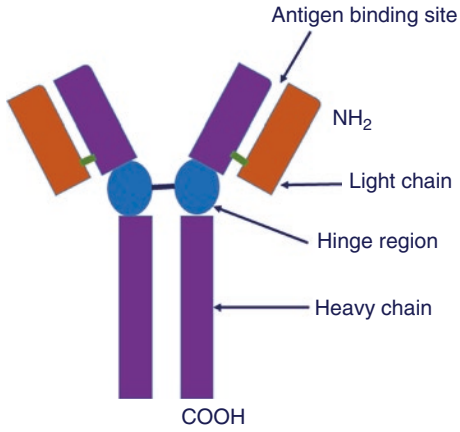

Fig. 16.2 Schematic diagram of immunoglobulin molecule. The antibody is a Y-shaped structure, and the two tips of the Y are known as antigen-binding site of the immunoglobulin

Polyclonal Antibody Polyclonal antibody is generated from the different B lymphocytes in response to the different epitopes of a single antigen. As they are generated from the different clone of B cells, these antibodies are known as polyclonal. There is a chance of batch-to-batch variation in case of polyclonal antibody.

The differences between the two types of antibodies are highlighted in Table 16.1.

Affinity Affinity represents the strength of the binding capacity of the antigenic epitope with the corresponding site of the antibody. It is actually the three-dimensional fit of the epitope site of the antigen with antibody.

Avidity Avidity means the overall functional strength of binding capacity of antibody and antigen complex. The polyclonal antibody reacts with multiple epitope sites of the antigen, and therefore the overall strength of antigen-antibody complex is strong. The avidity of an antibody depends on these factors:

1. *Valency*: The more valency of the antibody, the greater is the avidity.
2. *Affinity*: The affinity between the individual epitope of the antigen and the corresponding antigen-binding site of the antibody.

Table 16.1 The differences between monoclonal and polyclonal antibody

Monoclonal antibody	Polyclonal antibody
High production cost	Low production cost
Specialized training is required to produce	No special training is required to produce
Directed to a particular epitope of an antigen	Directed to multiple epitopes of an antigen
No variation of batch to batch	May vary from batch to batch
High specificity	Low specificity
Less robust of detection as the antibody is directed to a single epitope	More robust in detection as the antibodies are directed to multiple epitopes

3. *Structural arrangement*: Three-dimensional structural arrangement of antigen and antibody.

Antibody Specificity The antibody specificity indicates the precise detection of specific epitope of the antigen by the antibody. A particular antigenic determinant site (epitope) can be present in more than one antigen, and therefore a single antibody may react with different antigens.

Sensitivity This is related to the detection of the relative amount of antigen by the antibody in a particular technique. The highly sensitive technique detects low amount of antigen, and the relative intensity of the signal of antigen-antibody reaction is much strong.

16.2 Detection System

It is not possible to detect antigen-antibody reaction by light microscope. Therefore suitable detection or visualization system is necessary for the demonstration of such reaction. The different types of detection systems are highlighted below:

Direct Method In the direct method, the primary antibody is directly tagged with an enzyme or fluorescence (Fig. 16.3). The antibody should be specific for the particular antigen otherwise non-specific staining may occur.

Advantage:

1. Rapid and simple method

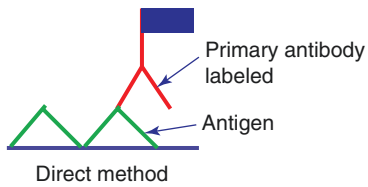

Fig. 16.3 Schematic diagram of direct immunostaining. In this method the primary antibody is directly tagged with an enzyme or fluorescence

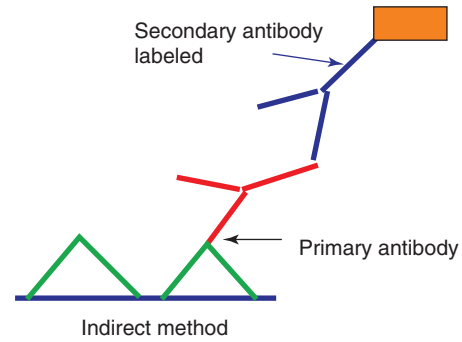

Fig. 16.4 Schematic diagram of indirect immunostaining. In this method, a labelled secondary antibody is used against the primary antibody

Disadvantages:

1. Different primary antibody should be labelled differently for the antigen.
2. Low sensitivity.

Indirect Method In case of indirect conjugated method, the primary antibody is unlabelled. The secondary antibody is conjugated and is directed against the primary antibody (Fig. 16.4). The antigen-primary antibody-secondary antibody complex is visualized by a suitable chromogen.

Advantages:

1. A single conjugated secondary antibody can be used against different primary antibodies.
2. Higher dilution of primary antibody can be used.
3. Large amount of secondary antibody can be easily produced against the primary antibody.
4. For negative control, the primary antibody can be omitted.

16.2.1 Peroxidase-Antiperoxidase Method (Fig. 16.5)

Peroxidase-antiperoxidase reagent is an immune complex substance. It consists of horseradish peroxidase antigen and antibody against horseradish peroxidase. Here a secondary bridging antibody is used between the primary antibody and peroxidase-antiperoxidase complex. The primary antibody and peroxidase-antiperoxidase reagents are

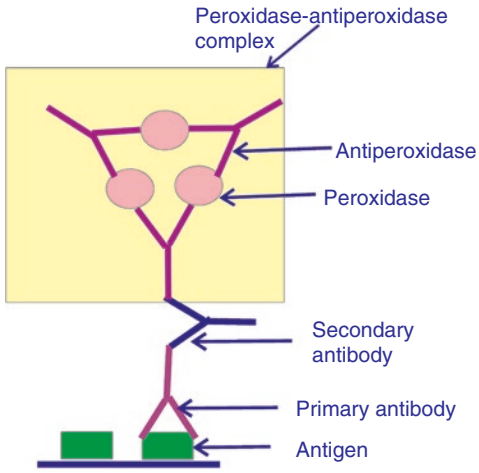

Fig. 16.5 Schematic diagram of peroxidase-antiperoxidase immunostaining. In this method, a secondary bridging antibody is used between the primary antibody and peroxidase-antiperoxidase complex

from the same species, whereas the secondary linking antibody is from a different species. This secondary antibody is usually highly specific against the primary antibody and the antibody present in the peroxidase-antiperoxidase complex.

Advantage:

1. High degree of sensitivity: peroxidase-antiperoxidase method is 1000 times more sensitive than the indirect conjugated method.

16.2.2 Avidin and Biotin Method

The biotin has the high affinity for avidin. In this method the secondary antibody is tagged with biotin. Now avidin conjugated with horseradish peroxidase is used. The biotinylated secondary antibody is tightly bound with the peroxidase-conjugated avidin (Fig. 16.6).

Advantage:

1. Rapid and sensitive method

Disadvantages:

1. Tissue may contain endogenous biotin which may react with the avidin. Therefore there is a chance of false-positive reaction.

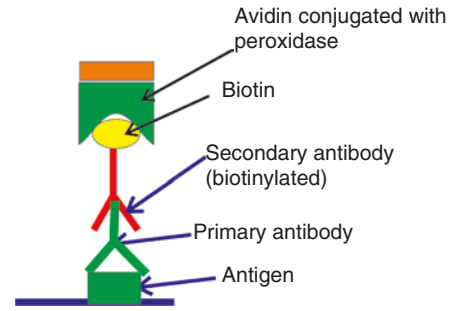

Fig. 16.6 Schematic diagram of avidin-biotin immunostaining. In this method the secondary antibody is tagged with biotin, and avidin conjugated with horseradish peroxidase is used. The biotinylated secondary antibody tightly binds with the peroxidase-conjugated avidin

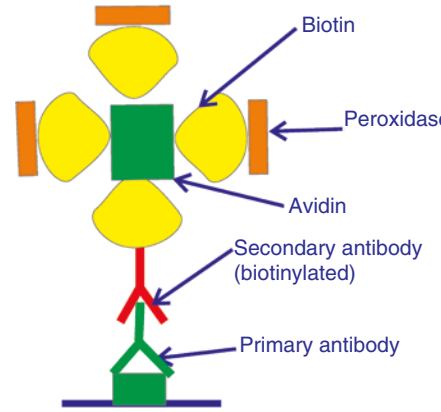

Fig. 16.7 Avidin-biotin-conjugated staining. In this method, a preformed complex of avidin-biotin and horseradish peroxidase is used

2. The affinity of the biotin and avidin may vary widely in different batches. This may significantly affect the sensitivity and reproducibility of the test.

16.2.3 Avidin and Biotin Conjugated Procedure

This is the modification of the above-mentioned procedure to further increase the sensitivity of the test. Here a preformed complex of avidin-biotin and horseradish peroxidase is used (Fig. 16.7). The presence of multiple molecules of horseradish peroxidase enhances the visualization reaction. The steps are such:

- Primary antibody
- Biotinylated secondary antibody
- Preformed complex of avidin-biotin and horseradish peroxidase

Advantage:

1. Highly sensitive

Disadvantage:

1. Endogenous biotin may cause false-positive reaction.

16.2.4 Biotin-Streptavidin Method

In this system the avidin is replaced by the tetrameric antibody streptavidin that is directly conjugated with enzyme. The streptavidin molecule has very high affinity against biotin. The biotin-streptavidin complexes give better amplification and detection than avidin-biotin complex.

Advantages:

- Streptavadin does not cross react with the lectin-like substances.
- The enzyme is more stable and can be stored for longer duration as this is directly bound with streptavidin.
- There is no non-specific electrostatic binding with streptavidin as its isoelectric point is near to neutrality.

16.2.5 Alkaline Phosphatase-Antialkaline Phosphatase Method [5, 6]

In alkaline phosphatase-antialkaline phosphatase method, we use a complex of alkaline phosphatase-antialkaline phosphatase (APAAP). The secondary antibody or linking antibody is used to bridge the primary antibody and APAAP complex (Fig. 16.8). Similar to peroxidase-antiperoxidase method, here also the primary antibody and antibody in the APAAP complex are both from the same species, whereas the secondary linking antibody is from a different species.

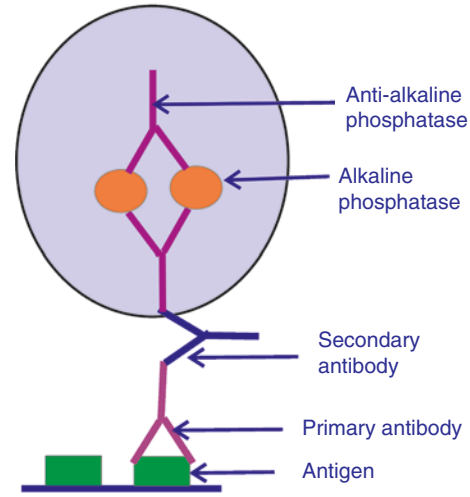

Fig. 16.8 Alkaline phosphatase-antialkaline phosphatase method. In this method, a complex of alkaline phosphatase-antialkaline phosphatase (APAAP) is used. The secondary antibody or linking antibody is used to bridge the primary antibody and APAAP complex

Advantages:

1. It is used in cases where the tissue contains high quantity of endogenous peroxidase such as in the bone marrow, lymph node, etc. [5]. It is often used in dual immunostaining such as APAAP and peroxidase-antiperoxidase staining.
2. APAAP method provides distinct bright red colour which is easy to identify compared to conventional peroxidase stain.
3. APAAP is stable for long duration.

16.2.6 Polymer-Based Labelling Method

In this technique a polymer backbone is used. This may be dextran, polypeptide or dendrimer polymer. The dextran polymer is often used as a polymer backbone. The large number of enzyme molecules (at least 100 peroxidase) and more than 20 secondary antibodies are conjugated within this dextran polymer (Fig. 16.9). At first

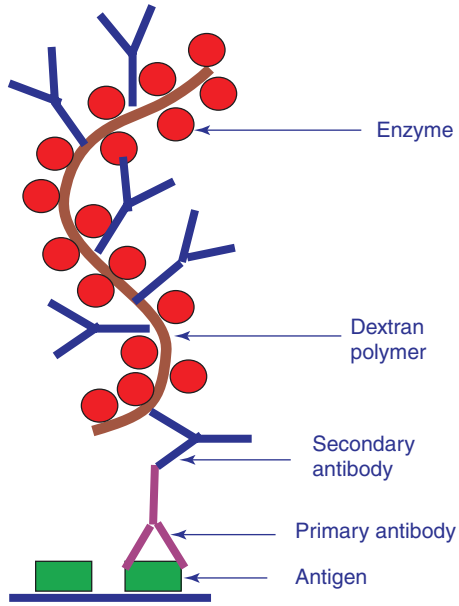

Fig. 16.9 Polymer-based labelling method. In this technique a polymer backbone (dextran) is used, and a large number of enzyme molecules and secondary antibodies are conjugated within the polymer. The primary antibody followed by dextran-enzyme secondary antibody complex is applied to enhance the sensitivity

primary antibody is applied followed by dextran-enzyme secondary antibody complex [7].

Advantages:

1. This is simple and rapid two-step technique.
2. Compared to avidin-biotin technique, this is more sensitive because the large number of enzymes takes part in a single binding of primary and secondary antibody.
3. No background staining as it bypasses biotin and avidin bindings.

16.2.7 Catalysed Signal Amplification (Tyramine Signal Amplification)

Catalysed reporter deposition technique is also known as tyramine signal amplification (TSA) method [8]. This technique exploits the catalytic property of HRP in the presence of hydrogen peroxide. Biotinylated tyramine is used in the presence of HRP and hydrogen peroxide. The HRP converts the biotinylated tyramine to reactive biotinylated tyramide. This activated biotinylated tyramide further reacts with tyrosine in the amino acid of the tissue and deposits biotin. This biotin is deposited only in the antigen-antibody reaction site. This biotin is visualized by avidin-biotin technique (Fig. 16.10).

Steps:

- Do initial horseradish peroxidase-conjugated system.
- Add biotinylated tyramine solution.
- Wash.
- Add horseradish peroxidase-conjugated streptavidin.
- Add chromogen (DAB) to visualize the reaction.

The comparison of different visualization techniques in immunohistochemistry is highlighted in Table 16.2.

16.3 The Sample of Tissues for Immunocytochemistry

16.3.1 Histopathology

- Formalin-fixed paraffin-embedded tissue
- Frozen section

16.3.2 Cytology

- Cell block tissue
- Smear from liquid-based cytology
- Direct cytology smear: imprint or FNAC smear
- Cytospin smear

16.3.3 Sample Collection

Histopathology

Formalin-fixed paraffin-embedded (FFPE) tissue: This is the most popular sample for IHC. Presently most of the antibodies work on FFPE tissue section.

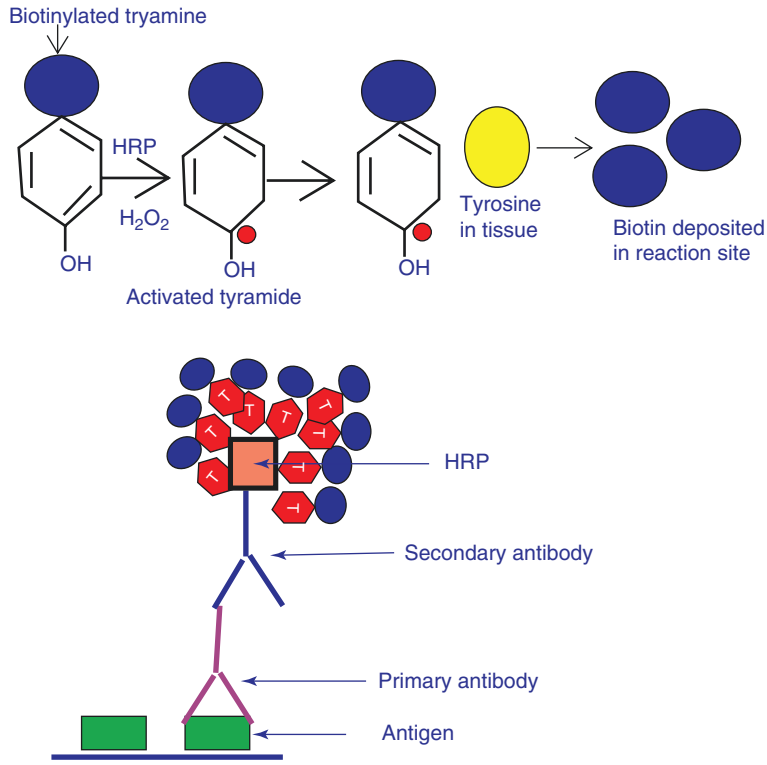

Fig. 16.10 Tyramine signal amplification (TSA) method. In this technique biotinylated tyramine is used in the presence of HRP and hydrogen peroxide. The HRP converts the biotinylated tyramine to reactive biotinylated tyramide that further reacts with tyrosine in the amino acid of the tissue and deposits biotin. This biotin is deposited only in the antigen-antibody reaction site. This biotin is visualized by avidin-biotin technique

Table 16.2 Comparison of different visualization techniques in immunohistochemistry

Name of technique	Primary antibody	Secondary antibody	Visualization system	Advantages	Comments
Peroxidase-antiperoxidase method	Primary Ab and antibody against HRP are of same species	Different species than that of primary Ab. This is a linking antibody	HRP antigen and Ab against HRP complex along with DAB used as chromogen	High degree of sensitivity	Unsuitable for bone marrow/ blood smear that contains lot of endogenous peroxidase
Avidin and biotin method	Primary Ab	Biotinylated secondary Ab is tagged with biotin	Peroxidase-conjugated avidin and a substrate for peroxidase	Rapid and sensitive	Endogenous biotin in the tissue may react with the avidin
Avidin- and biotin-conjugated procedure	Primary Ab	Biotinylated secondary Ab	Preformed complex of avidin-biotin and horseradish peroxidase	Highly sensitive	False positivity due to endogenous biotin
Biotin-streptavidin method	Primary Ab	Biotinylated secondary Ab	Streptavidin is directly conjugated with enzyme	• No cross reaction • More stable	
Alkaline phosphatase-antialkaline phosphatase method	Primary Ab and Ab against alkaline phosphatase are of same species	Secondary Ab	Alkaline phosphatase-antialkaline phosphatase complex	• Used in tissue containing high quantity of endogenous peroxidase • Stable • Bright red colour is easy to identify	Dual immunostain with peroxidase-antiperoxidase method is possible

(continued)

Table 16.2 (continued)

Name of technique	Primary antibody	Secondary antibody	Visualization system	Advantages	Comments
Polymer-based labelling method	Primary Ab	Secondary Ab	Polymer backbone conjugating with large number of enzyme molecules and more than 20 secondary Abs	• Simple and rapid two steps technique • More sensitive because the large number of enzymes takes part in a single binding of primary and secondary Ab • No background stain	
Tyramine signal amplification	Primary Ab	Secondary Ab tagged with HRP	Biotinylated tyramine reacts in the presence of HRP and hydrogen peroxide to generate activated biotinylated tyramide that reacts with tissue tyrmine	• Good sensitivity	• Time consuming • Non-specific background stain may appear

Ab antibody, *HRP* horseradish peroxidase

Frozen section: The use of frozen section for IHC is now discouraged.

Cell Block The cell block from the cytology material is preferable in case of cytology sample. Any specimen can be used for cell block except the specimen with very low cellularity.

Advantages of cell block:

1. Multiple sections available.
2. The tissue can be further used for archival use.
3. Minimum background staining.

Liquid-Based Cytology Multiple smears can be made by liquid-based cytology (LBC). The smears are small and contain good number of representative cells.

Advantages:

- Clean background
- Concentrated cells in a small area
- Multiple smears

Direct Smear The smears from the FNAC or other sample are made directly and fixed immediately. The direct smear gives poor quality of the immunostaining as there may be severe background artefact after immunostaining.

Advantages:

- No extra sampling
- No wet preparation

Disadvantages:

- Background staining artefact
- Limited antibody panel

Cytospin Preparations Multiple wet-fixed slides can be prepared from cytospin preparation of CSF, effusion or FNAC samples for the application of a panel of antibodies for ICC. Gross blood-mixed or mucus-mixed materials are unsuitable for cytospin. Background artefact may be seen in cytospin preparation, but overall cytospin preparation gives good result for ICC.

Advantages:

- Multiple smears can be made.
- Panel of antibodies possible.
- Overall good result.

Disadvantages:

- Specimen not fit for bloody and mucoid materials
- Difficult on scanty cellularity
- Best choice for lymphoid neoplasm

16.3.4 Fixation

Appropriate fixation of the tissue/cell is mandatory to get good IHC.

Buffered Neutral Formalin Buffered neutral formalin (10%) is still now the best fixative for tissue and cytology sample.

Advantages:

- Cell morphology is well preserved
- Cheap
- Sterilizes the specimen from bacteria
- Prevents diffusion of protein from the cell by cross-linking the protein

Disadvantages:

1. Buffered formalin is not good for RNA-based IHC. For RNA stain PAX gene, RCL2 or Z7-fixative is suggested [7]. PAX gene fixative gives excellent staining quality for RNA-based IHC [9].
2. Cross-linking of proteins by formalin hinders the access of the antibody to the epitope of the antigen. Therefore the antigen retrieval is often necessary for IHC on formalin-fixed tissue [10].

Others Commercially available fixatives are used in LBC preparations and they give equally good results. Various other fixatives are ethanol, methanol and acetone which are used in cytology. Alcohol fixative is not suitable for the demonstration of Her-2/neu oncoprotein, oestrogen and progesterone receptors, and buffered formalin solution is the most desirable fixative in this context.

16.3.5 Antigen Retrieval

Formalin-fixed tissue often gives inconsistent results of IHC. In real life it is very difficult to replace formalin as a tissue fixative. Enzymatic digestion of the tissue also does not always provide good result. Therefore there is a great need to have antigen retrieval in FFPE tissue. The term "antigen retrieval" (AR) means the recovery of the antigenicity of the tissue which is unmasked at the time of formalin fixation [10]. Fraenkel-Conrat et al. showed that the reaction between formalin and tissue is to some extent reversible if heat is applied [11]. Based on this theory, subsequently different studies have shown that the AR is possible by heating the tissue [12, 13].

The important factors that control the AR:

1. *Heating temperature and time period*: There is an inverse correlation between the heating temperature and time needed for AR. A test battery approach may settle the optimum temperature and time period for successful AR. However, low temperature (around 90 °C) with prolonged heating period helps in the perseveration of tissue morphology.
2. *pH of the AR solution*: Majority of the antibodies work well if pH is used around 7.4.

16.3.6 Microwave Retrieval

This is the most effective and popular method of AR [14]. It is always better to standardize the technique to get the consistent result. The following factors may need attention:

1. *Wattage*: The range of wattage of the microwave oven can be 750–800 W.
2. *AR buffer solution*: Usually 0.01 M citrate buffer of pH 6 gives good result.

3. *Volume of AR buffer solution*.
4. *Number of slides* in individual container.
5. *Thickness of tissue section*: Thinner tissue section (3 μm) requires less time.
6. *Type of antigen*: Nuclear antigen takes longer time for AR.

Requirements:

- Microwave oven
- Plastic container for incubation (microwave oven resistant)
- Plastic slide holder (microwave oven resistant)
- AR solution
- Silanized slides
- Tris-buffered saline
- Gloves, etc. for personal protection

Steps:

- At first remove paraffin and rehydrate the tissue section.
- Arrange the slides in a microwave oven-resistant plastic holder.
- Keep the holder with slides in the plastic container filled with AR solution. The solution should be adequate in amount to immerse the slides completely.
- Cover the container with a lid.
- Set the microvan for 10 min time (750–850 W).
- Remove the container and slides.
- Keep it cool for 20–30 min in room temperature.
- Wash the slides in distilled water.
- Keep the slides in Tris-buffered saline.

Warnings:

- Never allow the slides to dry.
- Never use metallic container or holder.

16.3.7 Pressure Cooker Heating

Pressure cooker heating provides uniform heating to all the slides.

Requirements:

- Pressure cooker: 4–5 L. Approximately 103 kPa will reach at a temperature of near about 120 °C
- AR solution
- Plastic slide holder
- AR solution
- Silanized slides
- Tris-buffered saline

Steps:

- At first remove paraffin and rehydrate the tissue section.
- Arrange the slides in a plastic holder.
- Put AR solution within the pressure cooker.
- Heat the system so that the AR solution is heated near to the boiling point.
- Keep the plastic holder with slides within the AR solution.
- Seal the lid of the cooker.
- Heat the cooker for 30 s to few minutes.
- Cool the cooker for 20 min.
- Open the lid.
- Transfer the slides in the Tris-buffered saline.

Precautions:

- The slides should be completely immersed in AR solution.
- The slides should not be dry under any circumstances.

16.3.8 Water Bath Heating

Water bath heating is the conventional procedure as water bath is easily available in most of the laboratories.

Requirements:

- Water bath. This should be temperature controlled.
- AR solution.
- Container to keep the AR solution.
- Plastic slide holder.
- AR solution.

- Silanized slides.
- Tris-buffered saline.

Steps:

- At first remove paraffin and rehydrate the tissue section.
- Pour good amount of AR solution to submerge the slides.
- Warm the container with AR solution in the water bath up to 98 °C.
- Now submerge the slide holder with slides within the already heated AR solution.
- Cover the lid of the container and incubate for 30 min.
- Take out the container and cool it.
- Rinse the slides in distilled water.
- Transfer the slides in the Tris-buffered saline.

16.4 Immunocytochemistry Technique

16.4.1 Control

In any IHC staining, it is extremely essential to have proper control because it validates the laboratory test. The control slides indicate the specificity of the test because it is essential to know that the antibody is reacted specifically to the specific epitope of the particular antigen and not with the other antigen.

Negative Control For negative control the primary antibody is omitted. In the absence of primary antibody, no staining reaction should occur.

Positive Control The known tissue section with the presence of antigen is used for positive control such as desmin stain wherein one can take uterine myometrial tissue as positive control.

The laboratories, which work on cell block only, should have adequate sections from cell block of each positive case. Paraffin-embedded sections should not be kept as a control for smear preparation.

16.4.2 Steps

The basic steps of immunocytochemistry are (Fig. 16.11):

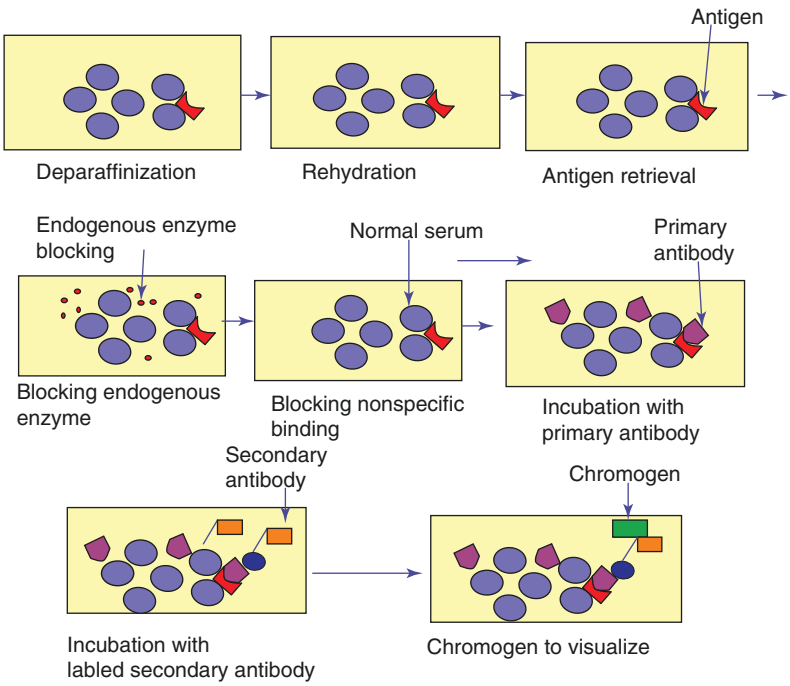

Fig. 16.11 Basic steps of immunocytochemistry is shown in this diagram

Table 16.3 Enzymes and blockage

Enzymes	Present in tissue	Blocking or prevention of reaction
Peroxidase	RBCs, polymorphs, histiocytes and hepatocytes	0.5% hydrogen peroxide in absolute methanol for 10 min
Alkaline phosphatase	WBC, liver, intestine, bone and placenta. However absent in formalin-fixed tissue	1 mM levamisole in 0.5 M HCl solution
Biotin	Liver, kidney, spleen	Polymer-based technique

Box 16.1: Background Non-specific Immunostaining

- Charged primary antibody reacts with oppositely charged molecules such as collagen.
- Fc receptors over cell surface of certain cells bind the antibody.
- Blocking:
- Incubate with 5% bovine serum albumin for 30–60 min.
- Block the Fc receptors by using Fab fragments.

1. *Antigen retrieval*: Described before.
2. *Blocking of endogenous enzyme*: In case of immunoperoxidase enzyme method, it is necessary to block the tissue endogenous peroxidase enzymes that are commonly present in RBCs, polymorphs and histiocytes. To block this endogenous peroxidase, the tissue should be kept in 0.5–1% hydrogen peroxide in absolute methanol for 10 min (Table 16.3). Alkaline phosphatase (AP) is present in the WBC, liver, intestine, bone, etc. When AP is used as a visualizing enzyme, it is necessary to block this enzyme. Usually AP is not survived in FFPE tissue; however, it may give trouble in frozen section. 1 mM levamisole in 0.5 M HCl solution is capable to block AP enzyme. Endogenous biotin may create problem in biotin-based visualization technique. Biotin is present in the liver, kidney, spleen, etc. Polymer-based method can effectively overcome this problem of endogenous biotin staining.
3. Blocking the background staining: Primary antibody are highly charged molecules, and they may bind by ionic and hydrophobic interaction with other reciprocally charged molecules such as collagen or other antibodies present in the serum (Box 16.1). Moreover Fc receptors are present over the surface of macrophages, lymphocytes, monocytes and polymorphs. These Fc receptors may also bind with the antibody and may produce background staining. Pre-incubation with 2% normal serum or 5% bovine serum albumin for 1 h may reduce the unwanted non-specific binding. The normal serum protein binds with the charged particles and neutralizes them. To block the Fc receptors instead of whole IgG, one can use Fab fragments.
4. *Incubation with primary antibody*: Optimal dilution of primary antibody is needed to avoid any false negative staining. Too much diluted antibody may fail to react with the tissue antigen. Therefore in case of any untested antibody, one should verify it with different series of dilution prior to its laboratory use. Only the dilution with intense clear staining should be considered as optimum dilution. In most of the commercially available antibody, the range of optimal dilution is already mentioned.
5. *Incubation with labelled secondary antibody*: Secondary antibody is usually labelled and directed against the primary antibody.
6. *Visualization*: Appropriate chromogen is applied to visualize the reaction.
7. *Counter staining*: Light counterstain is applied to visualize the nuclei and tissue architecture.
8. *Dehydration and mounting*: These are done after finishing all the previous steps.

Immunohistochemistry Protocol We generally follow this protocol in our institute.

- Deparaffinize by three changes in xylene.
- Rehydrate the tissue section by graded alcohol.
- Antigen retrieval: If necessary, by microwave treatment.
- *Endogenous peroxidase blocking*: Apply 3% hydrogen peroxide in methanol for 10 min (3 ml hydrogen peroxide in 100 ml methanol).
- Wash thoroughly twice by TBS 3 min each.
- *Blocking non-specific sites*: Apply 3% bovine serum albumin directly on the smear for 30 min.
- *Primary antibody*: Apply primary antibody with proper dilution in TBS with 1% bovine serum albumin for 30 min.
- Wash thoroughly twice by TBS for 3 min each.
- *Secondary antibody*: Apply secondary antibody conjugated with HRP or AP for 30 min (or biotinylated secondary antibody).
- Wash thoroughly twice by TBS for 3 min each.
- *Chromogen*: Apply appropriate chromogen and incubate for 3–10 min. Check under microscope after 3 min.
- Rinse in water.
- Counterstain: By haematoxylin for 10 s.
- Dehydrate in alcohol.
- Clear in xylene.
- Mount in DPX.

16.4.3 Chromogen

For HRP: 3,3′-Diaminobenzidine (DAB) is used.

Preparation:

10 mg DAB

10 µl hydrogen peroxide

10 ml Tris-buffered saline (TBS)

For AP: AP substrate solution: Fast red kit is used.

Tris-buffered saline

Tris base	61 g
NaCl	90 g
Distilled water	1000 ml

16.5 Troubleshooting in Immunocytochemistry

The common problems of IHC have been highlighted in Table 16.4.

Table 16.4 Troubleshooting in immunocytochemistry

Result and possible causes	
No stain • No stain or very little stain in both positive and test slide • No background stain	
Possible causes	Remedies
Defective primary or secondary antibody	• Expiry date may be over. Use fresh reagents • Defective storage of the antibodies
Forget to add primary antibody, secondary antibody or substrate material	• Repeat the procedure
Primary antibody is very much diluted and is almost absent	• Concentrate the primary antibody
Chromogen and substrate improperly used	• Use proper chromogen and substrate and repeat the test
Overstain by the counterstain	• Give less time for counterstain
Insufficient deparaffinization	• Give longer time in xylene and use fresh xylene
Very little or no antigen present in the tissue	• Use a highly sensitive IHC technique
Antigenic epitope site is destroyed during paraffin embedding	• Use wax of melting point below 60 °C
No stain in test section but Positive control is stained properly	
Too much fixation or improper fixation	Take precaution on fixation time and fixative solution
Necrotic or crushed tissue	Take better sample from the viable area
Background staining	
Both negative and positive controls are stained	
Secondary antibody is cross-reacting with the background substance	Apply the normal serum to block the cross-reacting antigen of the tissue

(continued)

Table 16.4 (continued)

Section or smear is dried during staining	• Take proper precaution to avoid drying of the tissue
Chromogen is incompletely dissolved	• Dissolve the chromogen properly
Positive control and test samples are showing background staining, but negative control is free from background stain	
Primary antibody is over concentrated	• Dilute the primary antibody properly
Only test sample showing background stain	
Over fixation and possibly the test sample and controls are differently fixed	• Standardize the proper fixation of the test sample

16.6 Applications

IHC is now an essential component in laboratory. It has widespread use in diagnosis, subclassification and prognosis of the tumors. IHC also helps in the identification of various infective organism of our body.

Box 16.2 highlights the major applications of IHC.

Box 16.2: Major Applications of Immunocytochemistry in Histology and Cytology

- Diagnosis
  - Classification malignant round cell tumors
  - Differentiating carcinoma, sarcoma, melanoma, etc.
  - Diagnosis of malignancy in effusion sample
  - Sub-classification
    Lung carcinomas
    Lymphoma
  - Diagnosis of soft tissue neoplasm
    Small round cell sarcomas
    Spindle cell tumors
    Pleomorphic sarcomas
- Prognosis
  - Cell proliferation analysis
  - Hormone receptors
  - Prognostic markers
- Therapeutic applications: Receptor studies for specific therapy
- Infective organisms to identify

16.7 Diagnostic Immunocytochemistry

16.7.1 Mesothelial Cells Versus Adenocarcinoma

IHC is often proved as very helpful to differentiate reactive mesothelial cells from adenocarcinoma [15]. Few important mesothelial markers are briefly outlined here:

16.7.2 Mesothelial Markers

Calretinin:

- Present in the mesothelial cells, steroid producing cells of ovary and testis, renal tubular cells and lipocytes.
- The sensitivity and specificity of calretinin are about 100% and 80%, respectively [16].

HBME-1:

- HBME-1 stains intensely around the periphery of the mesothelial cells.
- The specificity of HBME-1 is about 80%, and the majority of the mesothelial cells show strong positivity.

Wilms' tumor gene 1 (WT-1):

- This is a transcription factor isolated in the cells of the kidney.
- It is expressed in the cytoplasm and nucleus of the mesothelial cells.
- The WT-1 positivity is also seen in desmoplastic small round cell tumors and ovarian serous carcinoma.

D2-40:

- It is a relatively sensitive (85%) and specific (95%) marker of mesothelial cells.

Cytokeratin 5 and 6:

- Cytokeratin 5/cytokeratin 6 (CK5/CK6) shows diffuse cytoplasmic positivity in both benign and malignant mesothelial cells.
- CK 5/CK6 also stains other tumors such as squamous cell carcinoma, breast carcinomas, thymoma and salivary gland tumors [17].

16.7.3 Adenocarcinoma Markers in Effusion Fluid

BER EP4:

- BER EP4 antibody is highly specific and sensitive for adenocarcinoma.

Carcinoembryonic antigen (CEA):

- It is usually present in a scanty amount in normal epithelial cells and large amount in gastrointestinal adenocarcinoma and lung adenocarcinoma.
- Specificity of CEA is very high (100%) to differentiate mesothelial cells from adenocarcinoma.

MOC 31:

- MOC 31 is positive in all the cases of adenocarcinoma of the lung and many non-pulmonary adenocarcinomas.

Leu M1 (CD 15):

- Leu M1 (CD 15) specifically demonstrates carcinoma cells. Sensitivity of Leu M1 is low (29%).

16.8 Different Epithelial Markers

Cytokeratin:

- Depending on their molecular weights, cytokeratin is classified into 20 different types. The demonstration of specific cytokeratin is immensely helpful in pointing out the origin of an unknown metastatic tumor.
- Cytokeratin 7 and cytokeratin 20 phenotyping are particularly helpful to find out the primary tumor in metastasis. A combination of CK 7 and CK 20 often helps to locate the primary site of the malignancy (Fig. 16.12). Figure 16.13 and Table 16.5 illustrate the expression of CK and probable origin of tumors.

Epithelial Membrane Antigen (EMA) This complex glycoprotein is developed from milk fat globules and is unrelated to CK or intermediate filament. EMA is present in majority of the epithelial cells except certain tumors such as hepatocellular carcinoma and also adrenocortical carcinoma. Unfortunately, cells other than epithelial origin such as meningeal cell, mesothelial cells, certain lymphomas (anaplastic large cell lymphoma) and some sarcomas are also positive for EMA.

Carcinoembryonic Antigen (CEA) CEA is best demonstrated in the foetal epithelial cells. This glycoprotein is abundantly present in gastrointestinal and lung adenocarcinomas. Normal epithelial cells usually do not show CEA.

16.9 Mesenchymal Markers

Vimentin:

- Vimentin is usually expressed in mesenchymal cells such as fibroblast, endothelial cells, smooth muscle, etc. However, it has been demonstrated in epithelial neoplasms such as renal cell carcinoma and also mesothelial cells.

Desmin:

- Desmin is present in smooth muscle, skeletal muscle and cardiac muscle.

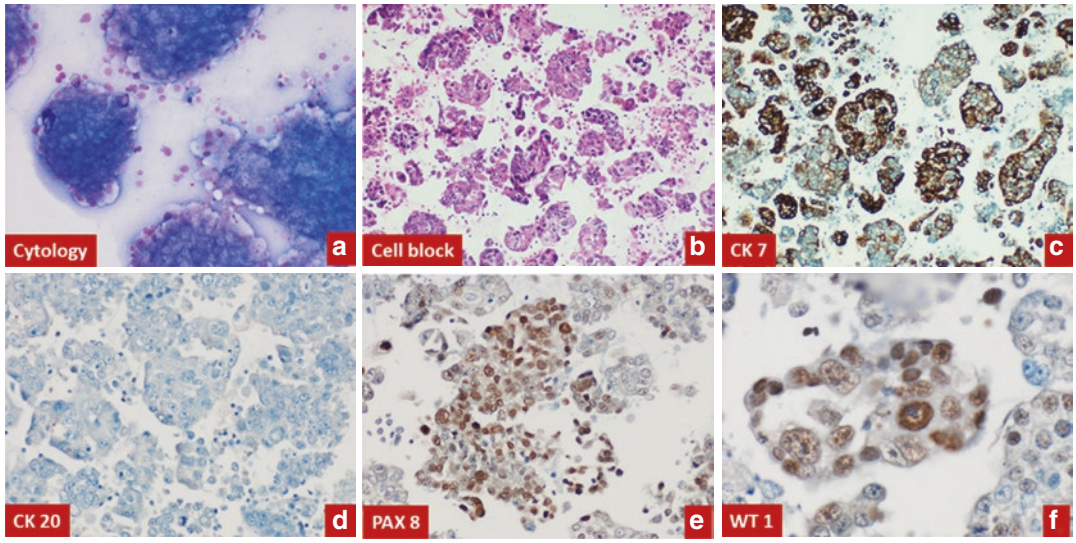

Fig. 16.12 Antibody panel helps to determine the exact sit of origin of the metastatic tumor. A 55-year-old female presented with massive ascites. Cytology smear (**a**) shows abundant tight ball like cluster, and cell block section shows (**b**) sheets of malignant cells with moderate nuclear enlargement and pleomorphism. The immunocytochemistry panel on cell block sections shows CK 7 (**c**), PAX 8 (**e**) and WT 1 (**f**) positivity and CK 20 negativity (**d**). The immunocytochemistry result indicates the ovarian origin of the carcinoma

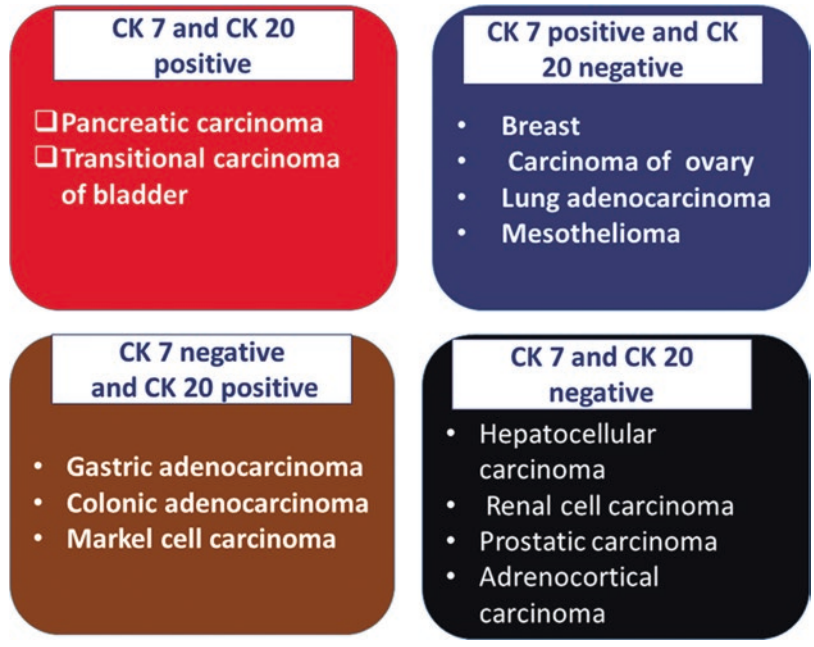

Fig. 16.13 The figure shows the various combinations of CK 7 and CK 20 positivity and the possible origin of the tumors

Table 16.5 Expression of CK and probable origin of tumors

Tumor type	CK 7	CK 20	CK 5/6
Adenocarcinoma of colon	–	+	–
Adenocarcinoma stomach	+	Occasional focal positive	–
Squamous cell carcinoma of the lung	–	–	+
Adenocarcinoma of the lung	+	–	–
Infiltrating duct carcinoma of the breast	+	–	Positive in basal type of carcinoma
Transitional cell carcinoma	+	Occasional focal positive	–
Ovarian adenocarcinoma (non- mucinous)	+	–	–
Renal cell carcinoma, clear cell	–	–	–
Pancreas and bile duct adenocarcinoma	+	Occasional focal positive	–

– negative, + positive

Actin:

- Actin is present in smooth muscle cells and myofibroblasts.

16.10 Neuroendocrine Markers

Chromogranin:

- Chromogranin is present in the neurosecretory granules in the neuroendocrine cells.

Synaptophysin:

- Synaptophysin is a marker of neuroendocrine and neuroectodermal tumors.

S-100 protein:

- S-100 protein is present in the nucleus and cytoplasm of glial and Schwann cells.
- It is also seen in melanocytes, Langerhans cells and myoepithelial cells and the neoplasm derived from those cells.

16.11 Lymphoid Markers

- The detailed immunophenotyping of lymphoid markers is very useful for identification of lymphoid cells and sub-classification of lymphomas.
- The essential lymphoid markers are highlighted in Table 16.6.

Table 16.6 Panel of antibodies for lymphoma subtyping

Cells and tumors	Markers
All lymphoid cells	CD 45
B cell	CD 19, 20, 23, 10
T cell	CD 3, 5, 4, 8
NK cell	Positive: CD 3, CD 56 Negative: CD 19, CD 10, CD 23
Hodgkin lymphoma	CD 15, CD 30

16.12 Melanoma Markers

HMB45:

- HMB 45 is a highly specific marker of melanoma.
- It also identifies neural crest-derived tumors, occasional carcinomas and immunoblastic lymphomas.

Melan A (Mart-1):

- This is a melanocyte-associated marker
- Melan A is also positive in peripheral nerve sheath tumor, angiomyolipoma and steroid producing cells of adrenal cortex [18].

16.13 Germ Cell Markers

Placental alkaline phosphatase (PLAP):

- PLAP is expressed in normal placenta.
- It is used to differentiate germ cell tumor and any other undifferentiated malignancy.

Human chorionic gonadotropin (HCG):

- HCG is normally secreted by the syncytiotrophoblast cell of the placenta.
- The beta subunit of HCG is hormone specific.

16.14 Site-Specific Antibody in Different Epithelial Malignancies

PSA and androgen receptor:

- Prostate-specific antigen (PSA) is positive in normal, hyperplastic and neoplastic prostate tissue.
- PSA is also positive in breast carcinoma and salivary gland tumors [19].

Androgen receptor:

- It is present in the nucleus of the prostate epithelial cells.

TTF:

- Thyroid transcription factor-1 (TTF-1) is present in follicular epithelial cells of the thyroid, bronchial epithelial cells of the lung and adenocarcinomas developed from those cells.

Oestrogen and progesterone receptors (ER and PR):

- ER and PR are present in the nucleus of the breast and endometrial adenocarcinomas.

16.15 Immunocytochemistry of Round Cell Tumor

Round cell tumors are often difficult to categorize by morphology alone particularly in cytology material. A panel of antibody is helpful to differentiate the different round cell tumor (Fig. 16.14). Table 16.7 highlights the basic panel to differentiate the different malignant round cell tumors.

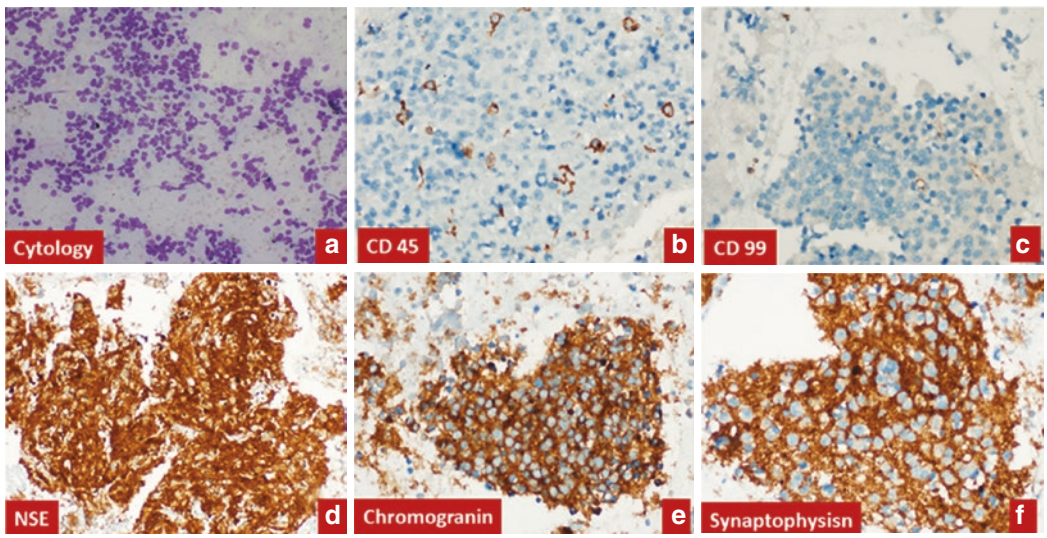

Fig. 16.14 Antibody panel to determine the type of a malignant round cell tumor. A 13-year-old male presented with 5 cm diameter right upper abdominal mass near the adrenal gland. The cytology smear (**a**) shows discrete monomorphic round cells. The cells are negative for CD 45 (**b**) and CD 99 (**c**) and positive for NSE (**d**), chromogranin (**e**) and synaptophysin (**f**). The result of the immunocytochemistry panel indicates the diagnosis of neuroblastoma

Table 16.7 Basic panel to differentiate the different malignant round cell tumors

Tumor type	CD 45	CD99	Myogenin	Pancytokeratin	Desmin	Chromogranin
NB	–	+	–	–	–	+
EWS/PNET	–	+	–	–	–	+
NHL	+	–	–	–	–	–
RMS	–	–	+	–	+	–
DSRCT	–	–	–	+	+	–
WT	–	–	–	+	+	–

NB neuroblastoma, *EWS* Ewing's sarcoma, *PNET* peripheral neuroectodermal tumor, *NHL* non-Hodgkin lymphoma, *RMS* rhabdomyosarcoma, *DSRCT* desmoplastic small round cell tumor, *WT* Wilms' tumor

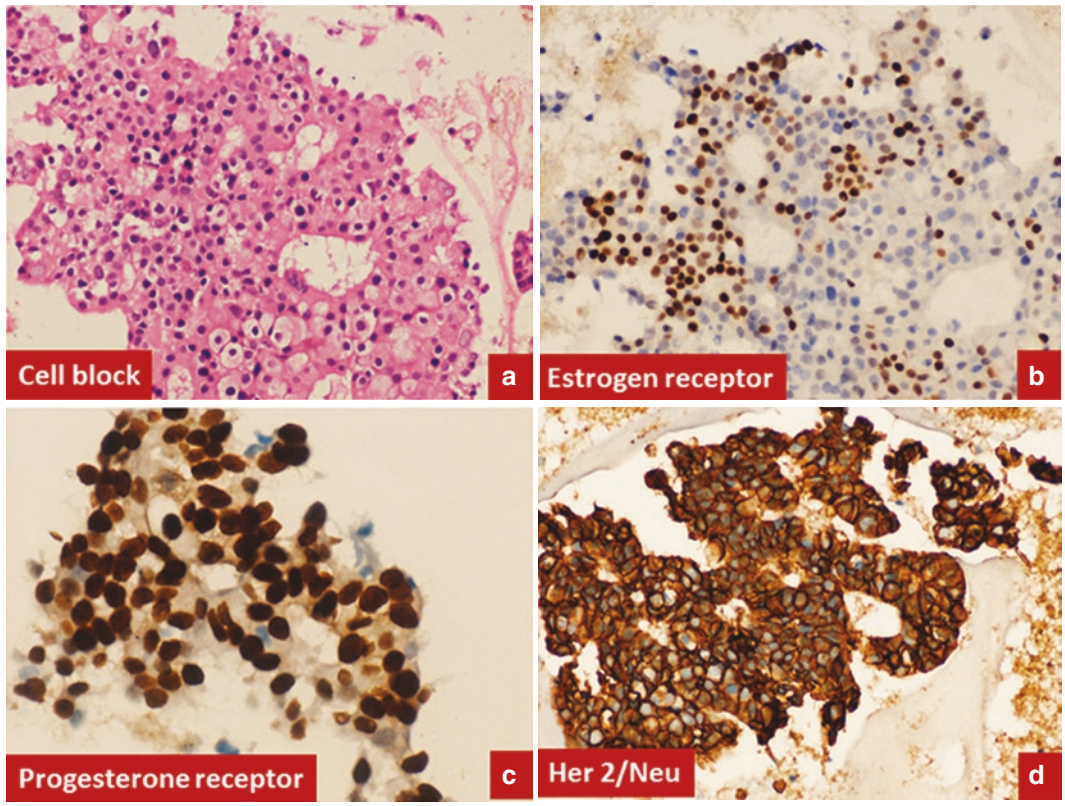

Fig. 16.15 The cell block of a case of infiltrating duct carcinoma of the breast (**a**). Oestrogen receptor (**b**), progesterone receptor (**c**) and Her-2/neu immunostain (**d**) were done that showed strong positivity

16.16 Immunocytochemistry for Therapy and Management

16.16.1 Breast Carcinoma

Various hormonal receptors along with Her-2/neu status of breast carcinoma are mandatory for the therapy and management of breast carcinomas (Fig. 16.15).

Oestrogen and progesterone receptors:

- ER/PR nuclear positivity is related to anti-oestrogen hormone therapy of breast cancer.

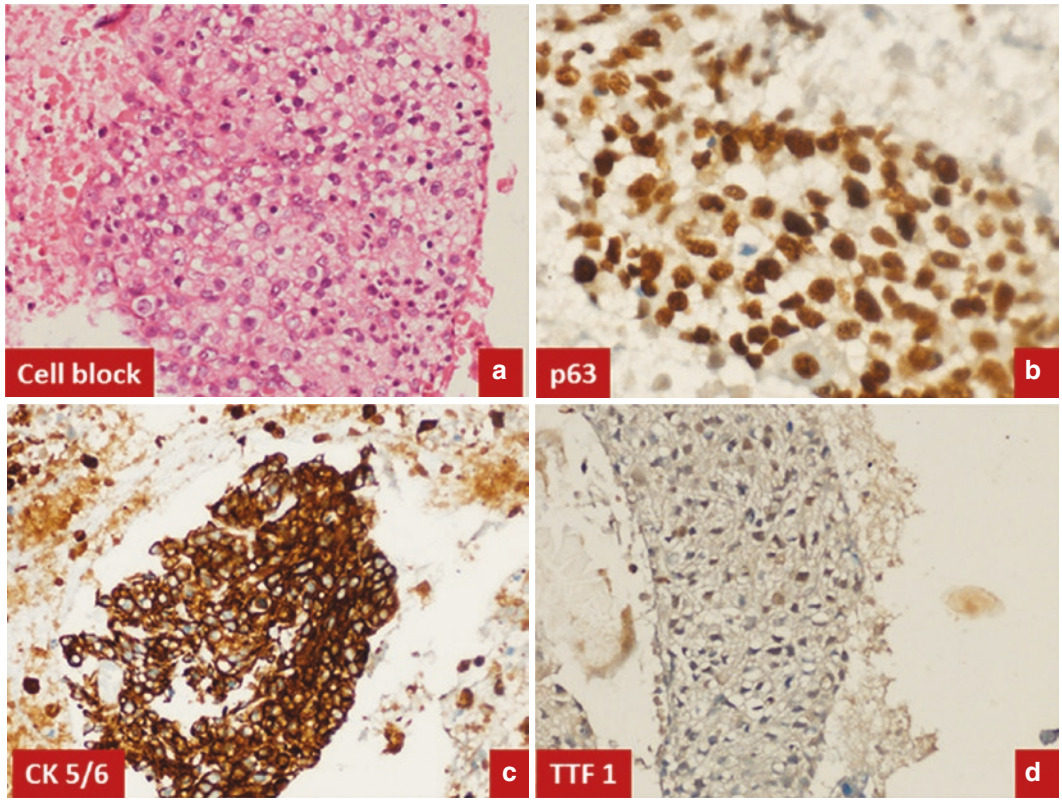

Fig. 16.16 A case of lung carcinoma (**a**) shows p63 (**b**) and CK 5/CK6 positivity (**c**) and TTF-1 negativity (**d**). This indicates the diagnosis of squamous cell carcinoma

Table 16.8 Markers of lung carcinoma

Type	p40	p63	TTF1	Napsin	CK 5/6	CK 7
Squamous cell carcinoma	+	+	–	–	+	–
Adenocarcinoma	–	–	+	+	–	+

+ positive, – negative

Her-2/neu:

- The status of Her-2/neu overexpression in breast cancer is helpful for anti-Her-2/neu antibody therapy.

16.16.2 Gastrointestinal Stromal Tumor

CD117 (C-Kit) Gastrointestinal stromal tumor with C-Kit mutation (high surface level of CD 117 expression) is responsive to tyrosine kinase-inhibiting agent (imatinib) therapy [20].

16.16.3 Lung Carcinoma

A panel of antibody is helpful to differentiate squamous cell carcinoma from adenocarcinoma of lung (Fig. 16.16). The cases of lung adenocarcinoma should be investigated for EGFR mutational changes for specific receptor inhibitory

drugs. Table 16.8 shows the basic panel for the differentiating adenocarcinoma from squamous cell carcinoma of the lung.

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17 Flow Cytometry: Basic Principles, Procedure and Applications in Pathology

17.1 Introduction

Flow cytometry (FCM) is the method by which the various characteristics of individual particle or cells are studied. FCM provides us very quick assessment of cell surface antigens, DNA content and intracellular proteins. The instrument is now used not only for research but also for routine clinical activities [1–3]. It is now a well-established technique for the diagnosis and classification of lymphoid neoplasms, identification of malignant cells in effusion cytology and other body fluids. Modern flow cytometer is now much more user-friendly and smaller in size.

17.2 Principle of Flow Cytometry

Single dissociated cells in liquid medium are essential for flow cytometry. The specific component of the cell is identified by the antibody tagged with a fluorescent dye. Similarly DNA can also be stained by a DNA stoichiometric dye. The single cells rapidly pass in front of a laser beam, and the laser beam of particular wave length hits the cell. The individual cells absorb the light and emit light of different wavelength. The emitted light is detected by the photomultiplier tube and is converted into a digital pulse. The intensity of the digital signal is stored in the computer and expressed in a relative scale known as channel. The result of the events is expressed as dot plot or histogram (Fig. 17.1).

17.3 Dye Used

17.3.1 Fluorochrome Dyes for FCM

What is Fluorescence When a fluorochrome compound absorbs quantum of light, the electron moves from lower orbit to the higher orbit, and compound remains in excited state. When the compound comes back to its ground state, the electron returns to its original orbit, and the compound emits the quantum of light of lower wavelength with a different colour (Fig. 17.2). The whole phenomenon is known as fluorescence. As the excited light wave and emitted light waves are of different wavelength and colour, the emitted light is easily visible and can be recorded.

Each fluorochrome dye compound has certain properties:

- Specific excitation spectrum of light
- Specific emission spectrum of light
- The quantum efficiency

The difference between the wavelength of excitation or absorption spectrum and emission spectrum is known as Stokes shift. The higher the value of the Stokes shift, the greater the separation of the excitation and emission spectrum.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_17

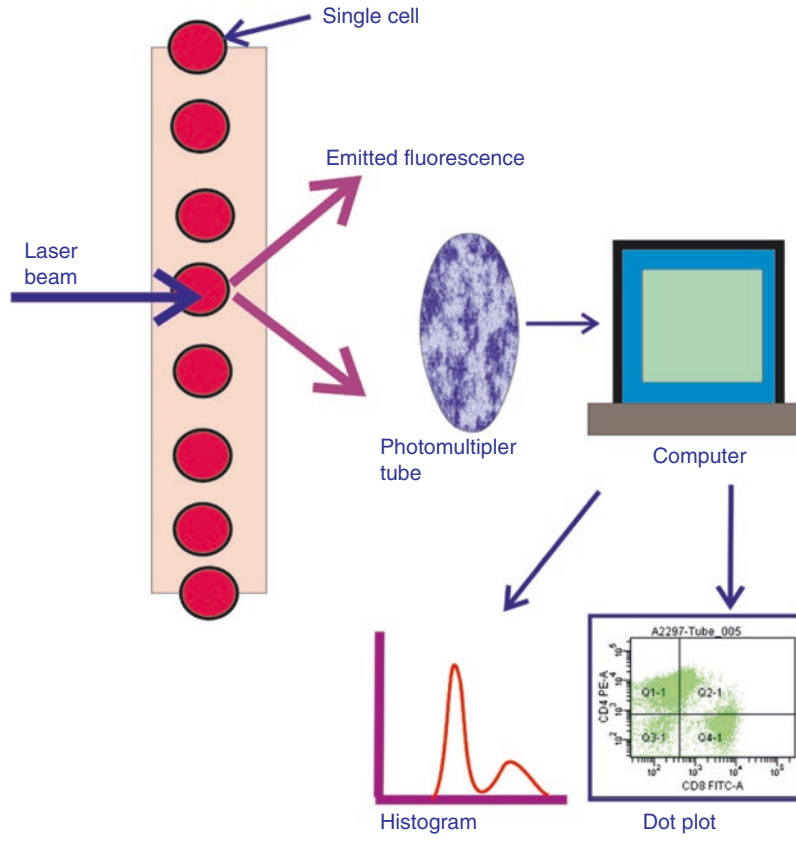

Fig. 17.1 Schematic diagram of basic principle of flow cytometry is highlighted in the picture. The single cells are hit by the laser beam of particular wavelength. The emitted light is detected by the photomultiplier tube and is converted into a digital pulse. The result of the events is expressed as dot plot or histogram

Box 17.1: Principle of Flow Cytometer

- Single cell suspension.
- The specific component of the cell is stained with a fluorescent-tagged antibody.
- DNA-binding dye binds with DNA stoichiometrically.
- A laser beam of particular wavelength hits the cell.
- The cell absorbs light and emits fluorescence.
- The emitted fluorescence is recorded with the help of the photodetectors.
- The electronic signal is converted into digital signals.
- The results are then displayed as histogram or two-dimensional dot plots.

17.3.2 Fluorochrome Dye for Nucleic Acid

The common dyes in DNA FCM include propidium iodide, ethidium bromide, Hoechst 33342 and diamidinophenylindole (Table 17.1).

Fluorochrome Dye for Antibody and Protein Various fluorochrome dyes are used for conjugating antibodies and protein. The most commonly used fluorochrome dye is fluorescein isothiocyanate (FITC). Table 17.2 highlights the excitation and emission spectrum of different commonly used fluorescein dyes.

Dye for RNA Content Measurement RNA content is measured with acridine orange, pyronin Y and oxazine 1.

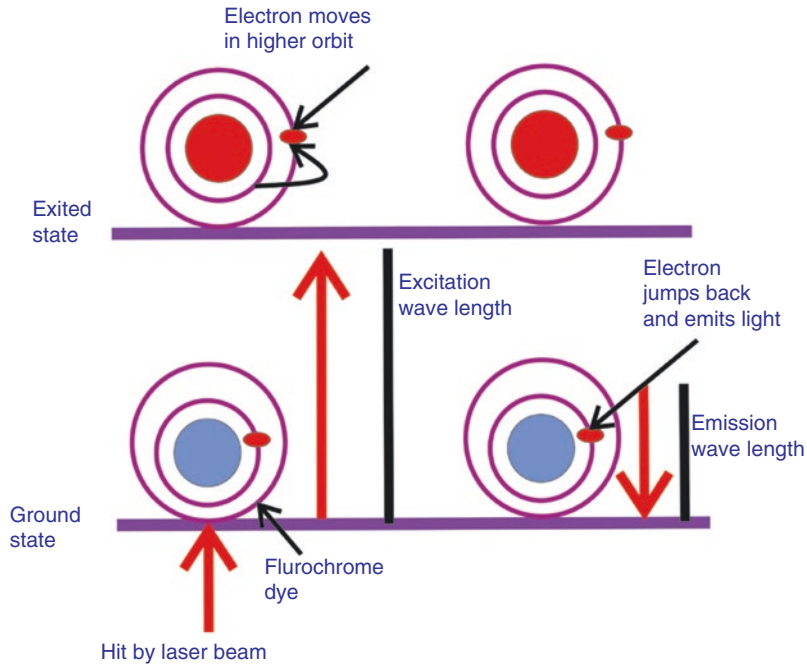

Fig. 17.2 The diagram shows the principle of fluorochrome dye activation. When a fluorochrome compound absorbs quantum of light, the electron moves from lower orbit to the higher orbit, and the compound remains in excited state. When the compound comes back to its ground state, the electron returns to its original orbit and the compound emits the quantum of light of lower wavelength with different colour

Table 17.1 Fluorochrome dye used in DNA

Flurochrome	Excitation maximum (nm)	Emission maximum (nm)
Propidium iodide	305, 540	620
Ehidium bromide	493	620
Hoechst 33342	350	461
Hoechst 33258	352	461
Diamidinophenylindole (DAPI)	359	461
Acridine orange	503	530 (DNA), 640 (RNA)

Table 17.2 Fluorochrome dye for conjugating with antibody

Fluorochrome	Excitation maximum (nm)	Emission maximum (nm)
Fluorescein isothiocyanate (FITC) 488	495	519
Phycoerythrin (PE)	496	576
Allophycocyanin (APC)	650	660
Rhodamine Red-X	570	590
Texas Red®	595	613
PE-Cy7® 488	566	778
Peridinin chlorophyll (PerCP)	477	678

nm nano micrometre, 10^{-9} m

17.4 Samples for Flow Cytometry

17.4.1 Cytology Samples

The various types of cytology specimens for FCM include:

1. Fine needle aspiration cytology materials: Lymph node, breast, lung, prostate, etc.
2. Exfoliative samples: Effusion fluid, CSF, bladder wash

The special *advantages of* cytology specimens (Box 17.2) over the histopathology tissue include:

1. Cytology samples are easy to process and require less effort for disaggregation and thereby single cell preparation is easier to make for FCM.
2. It is relatively easy to procure cytology sample.

Box 17.2: Advantages of Cytology Sample for FCM

- Easy to process
- Less effort to disaggregate the cells
- Easy to procure the sample
- Possible to study from the multiple areas of the tumor or lymph nodes
- Viable cells so functional studies are possible

Box 17.3: Citrate Buffer

- Sucrose: 85.3 g
- Trisodium citrate (Sigma): 11.8 g
- 50 ml of dimethyl sulfoxide in 100 ml of water
- pH keep at 7.6

3. Multiple areas of the tumor or lymph nodes can be collected for processing.
4. The different functional studies can be done as the cells are viable.

Collection The cytology samples can be collected in 1 ml of citrate buffer solution (Box 17.3). The sample can be processed immediately or may be kept at −70 °C to process later on.

17.4.2 Histology Samples

- Frozen section tissue
- Paraffin-embedded tissue

Histopathology sample needs thorough disaggregation for flow cytometry. In fact, paraffin-embedded tissue does not give good result on FCM.

17.4.3 Control

DNA FCM control: Lymphocytes from the peripheral blood samples of healthy individuals.

Flow cytometric immunophenotyping: The indications for use of controls are:

- Any suspicion of artefactual change
- To monitor the autofluorescence and non-specific binding in the sample

Negative control: Unlabelled processed sample may work as negative control.

With the help of commercially available fluorescein-labelled beads, one should do the setting of optics, fluidics and electronics. The specific company provides certain instructions and this should be obeyed strictly.

17.4.4 Sample Processing

DNA flow cytometry [1]

- Centrifuge the sample: 3000 rounds per minute for 3–5 min.
- Discard the supernatant fluid.
- Resuspend in citrate buffer.
- Prepare single cell suspension by repeated syringing through nylon mesh or by trypsinization.
- Keep the cell concentration in the buffer is kept as at least 2×10^6 cells per ml in 0.4 ml PBS (pH 7.4).
- Add 500 µl of solution A containing Triton X-100, RNAse and propidium iodide fresh solution (see below).
- Incubate for 30 min in room temperature in the dark.
- Run in flow cytometer within 2–3 h.

Stock solution of propidium iodide (PI)

• Propidium iodide (PI)	1 mg
• Water	2 ml

Store in 4 °C in the dark

Solution A

1. RNAse A	2 mg
2. Triton X-100	10 ml: 0. 1% (v/v)
3. Propidium iodide	0.40 ml of stock solution (500 µg/ml)

Make fresh solution

17.4.5 Flow Cytometric Immunophenotyping (FCI)

Direct stain:

- Centrifuge the sample: 3000 rounds per minute for 3–5 min.
- Discard the supernatant fluid.
- Re-suspend in citrate buffer.
- Prepare single cell suspension by repeated syringing through nylon mesh or by trypsinization [1].
- Keep the cell concentration in the buffer is kept as at least 2×10^6 cells per ml.
- Take in 100 µl in PBS (pH 7.4).
- Add 10 µl of labelled antibodies for 25 min in the dark place at room temperature.
- Wash the cells three times in PBS solution by 3000 rounds per minute for 3–5 min.
- Discard supernatant.
- Resuspend the cells in 500 µl PBS solution.
- Run in FCM.

Indirect staining procedure [2, 3] (Fig. 17.3):

- Collect sample in citrate buffer.
- Wash the cells in PBS (3000 rounds per minute for 3–5 min).
- Discard the supernatant and resuspend the cells in ice-cold PBS, 3% BSA, 1% sodium azide.
- Keep 100 µl solution of cells (1.5×10^6 cells/ml concentration of cells) into multiple small aliquots.

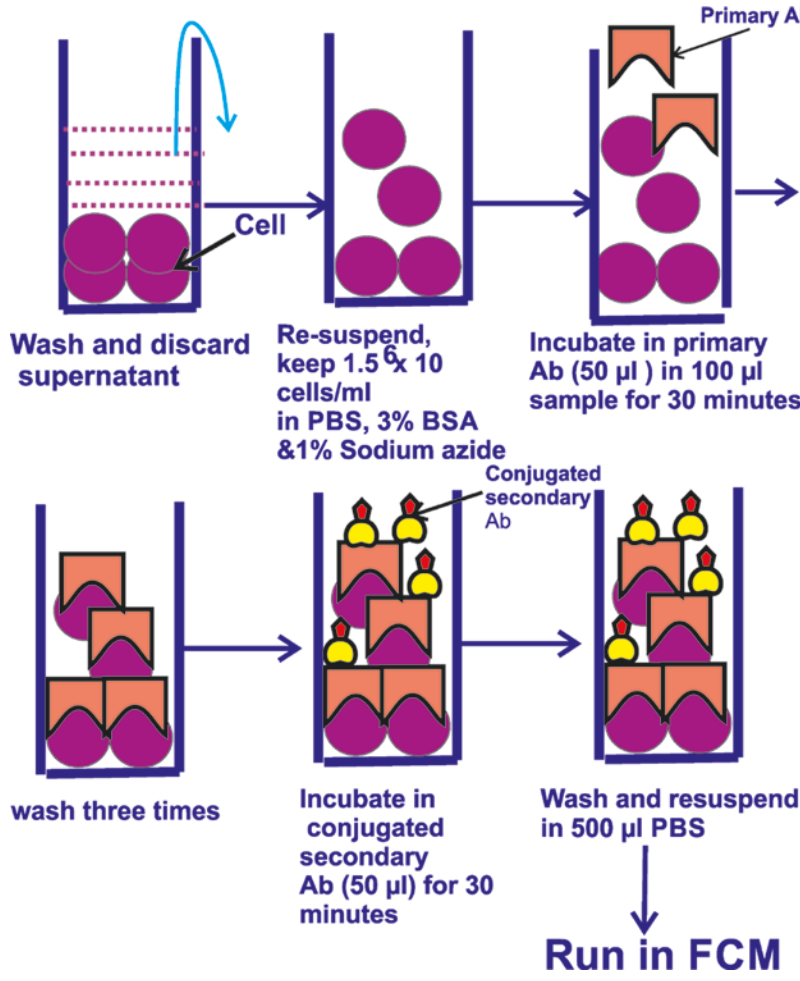

Fig. 17.3 Schematic diagram explains the steps of indirect immunostaining

- Take 100 µl solution of cells and incubate with 50 µl of primary nonconjugated antibody for 30 min at room temperature in the dark.
- Wash the cells in PBS.
- Add 50 µl of a conjugated secondary antibody.
- Keep for 30 min at room temperature.
- Wash three times in PBS.
- Discard the supernatant and resuspend the cells in 500 µl PBS.
- Run in FCM.

17.4.6 Data Acquisition [4]

- Mention clear labelling of the specific sample and the name of the antibody used.
- Mention approximate total number of events.
- Carefully monitor the fluidics.

Data interpretation

- At the time of reporting, it is essential to assess the overall quality of the sample: light scatter, fluidics, etc.
- Do the doublet exclusion in case of cell aggregation.
- Follow appropriate gating strategy.

Reporting

- Sample
  - Identify the sample number.
  - Type of sample.
  - Test to do.
- Details of patient's information:
  - Name
  - Sex
  - Age
  - Chief complaints
  - Clinical diagnosis
- Report of FCM
  - Percentage of cell of interest showing positive staining of the antibodies
  - Cell of interest showing negative staining
  - Detailed description of abnormal cell population: Antigenic expression, light scatter properties
  - Cell ploidy analysis: DNA ploidy patter, percentage of S-phase cells
  - Mention summary of the different findings, conclusions and further suggestions

17.5 Targets of Application

FCM can be used for quantitative measurement of various cellular characteristics (Box 17.4). Many of the targets of application of FCM are research oriented.

The most common applications of FCM in clinical laboratory include [5, 6] (Fig. 17.4):

Box 17.4: Measurement of Cellular Features

- DNA ploidy analysis
- Cell cycle analysis
- Intracellular and cell surface receptors
- Cell size
- Cell viability and apoptotic cells
- Enzyme activity: phosphatase, glucuronidase, etc.
- Metabolic studies: oxidative burst, intracellular pH, intracellular calcium
- Cellular protein, lipid, haemoglobin
- RNA content
- Mitochondrial function
- Physical phenomena: phagocytosis, pinocytosis, etc.

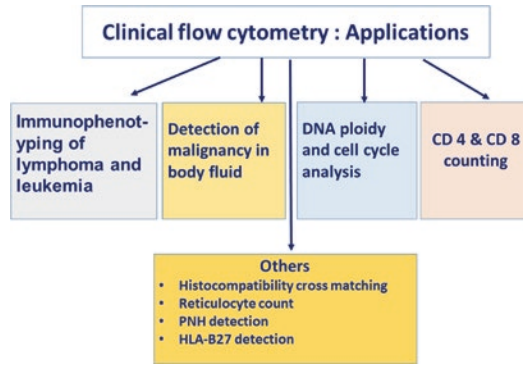

Fig. 17.4 Common applications of flow cytometry is highlighted in this diagram

- Immunophenotyping of lymphoma and leukaemia
- DNA ploidy analysis and cell cycle analysis
- Detection of malignancy in body fluid

In addition, FCM is used also for histocompatibility, cross matching, HLA-B27 detection, CD 4 and CD 8 counting for immunodeficiency, reticulocyte enumeration and PNH detection in immunology and haematology laboratories.

17.6 DNA Content and Ploidy Analysis

Basic principle:

- DNA-specific dye stochiometrically binds with the DNA of the nucleus.
- Emitted fluorescence from the dye-DNA complex is directly proportional to the DNA content of the nucleus.
- The data are displayed as DNA histogram.
- The majority of the normal cells contain diploid (2n) chromosome so they emit a certain amount of fluorescence represented by a channel number. This forms a single peak known as the diploid peak in DNA histogram.
- The cells in G_2M phase contain the double amount of DNA (4n) so they emit the double amount of fluorescence and are present in double channel number in the DNA histogram forming a small tetraploid peak.
- In between these two peaks, the cells have varying amount of DNA from 2n to 4n, and they are in synthetic (S) phase.
- Any peak, other than these two peaks is considered as an aneuploid peak.

The following information is obtained from DNA FCM:

1. Identification of aneuploid cell population
2. DNA index
3. Proliferative cell fraction (% of S phase)

DNA Ploidy The clone of cells containing the abnormal amount of DNA is known as aneuploid cells. In DNA histogram the aneuploid population will form a peak somewhere other than diploid peak.

The relative DNA content of the aneuploid population of cells is calculated by DNA index.

$$\text{DNA index} = \frac{\text{Mean channel number of aneuploid peak}}{\text{Mean channel number of normal diploid peak}}$$

The different types of aneuploidy are mentioned below:

Hyperdiploid Aneuploidy (Fig. 17.5) Here the aneuploid peak lies in between diploid and tetraploid peak (DNA index is in between 1 and 2).

Hypodiploid Aneuploidy (Fig. 17.5) Here the aneuploid cell population forms a peak left to the diploid peak as they contain less than the 2n amount of DNA (DNA index is lower than 1).

Tetraploid Aneuploid (Fig. 17.5) Aneuploid population of cells makes a peak on G_2M peak. It is often difficult to distinguish tetraploid

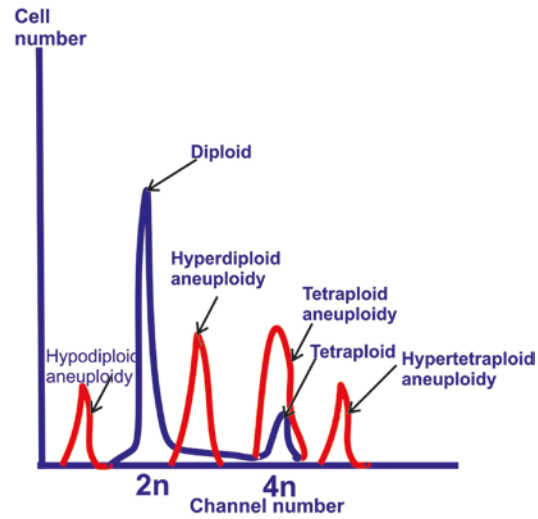

Fig. 17.5 Schematic diagram of different types of aneuploidy in DNA histogram

peak from normal G_2M peak. However, the presence of more than 20% cell population in this peak suggests a tetraploid aneuploidy (DNA index is 2).

Hypertetraploid Aneuploidy (Fig. 17.5) Here the aneuploid population of cells forms a peak beyond the G_2M peak as they have more than 4n amount of DNA peak (DNA index is greater than 2).

S Phase The S-phase fraction of cells has 2n–4n amount of DNA so they remain in between G_0G_1 and G_2M peak. The number of such cells can be calculated from the DNA histogram.

17.6.1 Clinical Application

DNA FCM provides usual information on the DNA ploidy and S-phase fraction of the tumor cell population. The presence of aneuploidy cell population and/or high S phase suggests a malignant lesion [1, 2]. However, we should keep in mind that malignant tumors may often show diploid cell population, and also uncommonly benign tumor may have aneuploidy population of cells.

17.6.2 Diagnosis

Effusions Diagnosis of malignancy only on the basis of DNA ploidy has limited use. DNA FCM along with staining of other markers (BER EP4, Leu M1, etc.) helps to identify adenocarcinomas in effusion fluid [7]. Ber-EP4 is an epithelial cell adhesion molecule. BER-EP4 monoclonal antibody is directed against the protein moiety of the glycopeptides of the epithelial cells. This molecule is expressed in the epithelial cell and its malignancies. As epithelial cells are not normally present in the effusion fluid, the presence of BER-EP4 positive cells in effusion usually indicates metastatic epithelial tumor. The sensitivity and specificity of BER-EP4 in detection of malignancies in effusion are about 80 and 90%, respectively [7].

Bladder Washings DNA ploidy estimation in the gated population of cytokeratin positive cell is helpful in the detection of malignancy in follow-up cases of urothelial carcinoma of the bladder [8].

CSF Due to low cellularity, DNA FCM has less diagnostic capability in the detection of malignancy in CSF. It has been demonstrated that the combined use immunophenotyping and DNA FCM is more helpful in the diagnosis of lymphoma in CSF [9, 10].

17.6.3 Prognosis of the Patients

DNA aneuploidy and high S phase are poor prognostic factors in various solid tumors such as bladder, prostate, ovarian and endometrial carcinomas [11].

17.7 Immunophenotyping

A large number of CDs have been described in different cells of lymphoid origin [12] (Table 17.3). Monoclonal antibodies against various CDs are now also available for the use of flow cytometric immunophenotyping (FCI) (Figs. 17.6, 17.7, 17.8 and 17.9). The antibodies

Table 17.3 CD markers of lymphoid cells

Cells	Markers
All lymphoid cells	CD 45
T lymphocyte	CD 2 CD 3
T helper cell	CD 4
T cytotoxic cell	CD 8
T regulatory cell	CD 4 CD 25
NK cell	CD 56
B lymphocytes	CD 19 CD 20
Plasma cell	CD 138
Plasmacytoid dendritic cell	CD 304
Stem cell and progenitor cell	CD 34 CD 117 CD 271

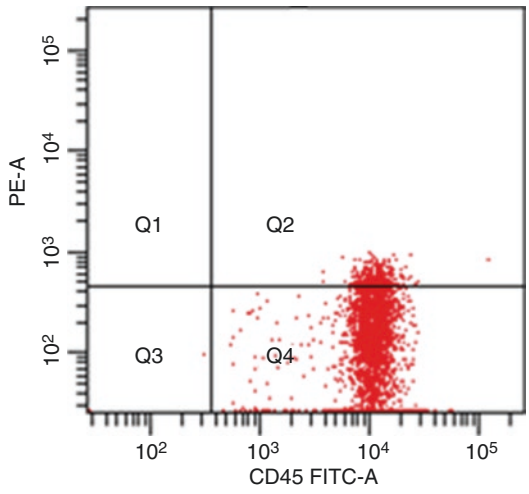

Fig. 17.6 Dot plot of CD 45 stain in flow cytometry

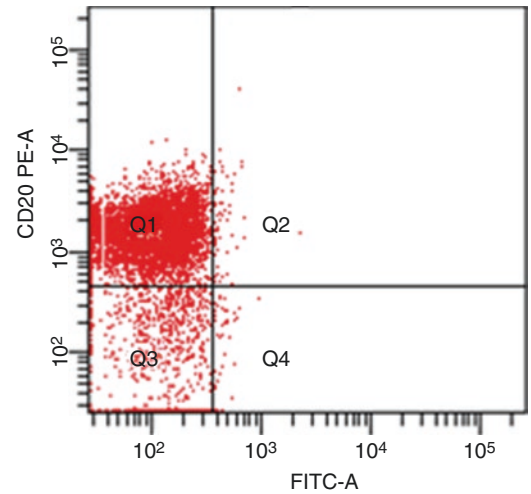

Fig. 17.8 Dot plot of CD 20 stain (B cell marker) in flow cytometry

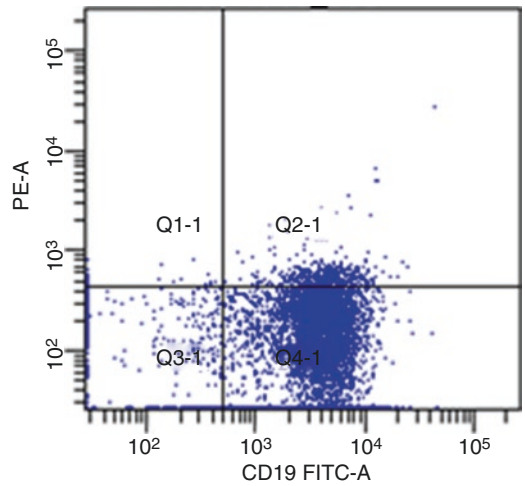

Fig. 17.7 Dot plot of CD 19 stain (B cell marker) in flow cytometry

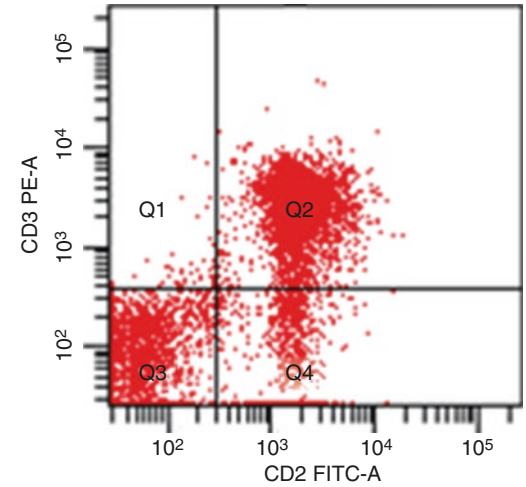

Fig. 17.9 Dot plot of CD 2 and CD 3 stain (T cell marker) in flow cytometry

are labelled by different fluorochromes of different emission spectrums. Therefore judicious use of the fluorochrome-tagged antibodies may help to perform multicoloured FCI. This has a great impact in application of FCI as one can study on the large number of CDs in a small volume of sample. The demonstration of various CD markers on the lymphoid cell surface helps in the diagnosis and also sub-classification of lymphomas.

Diagnosis:

- Most of the cases of lymphoma are monoclonal. So light chain restriction of either lambda or kappa chain indicates lymphoma (of B cell origin). In FCM the kappa/ lambda ratio more than 4:1 or more than 1:2 is usually considered as an evidence of monoclonality.

- Clonality demonstration in T cell NHL is difficult in FCM. However the aberrant expression of CD 2, CD 5 and CD 7 expression may suggest T NHL [13].

Sub-classification of Lymphomas

FCI can be done easily on FNAC material because the cells are discrete in cytology material. The advantages of FCI over immunocytochemistry are:

- It is a fast technique to analyse large population of cells.
- The various CD markers can be applied in small volume of aspirate.
- Dual expression of CD markers.
- Additional features such as DNA ploidy, cell cycle analysis, etc. can be done simultaneously along with immunophenotyping.
- Quantitation of antigen-positive cells is possible.

Figure 17.10 highlights the comparison of FCM immunophenotyping and immunocytochemistry.

17.7.1 Limitations of FCI

FCI bears certain limitations that include:

1. *No morphological correlation*: This is the greatest disadvantage of FCI. It is not possible to correlate morphological findings with the FCI data. Laser scanning cytometry overcomes this problem.
2. *Admixture of other material*: Benign reactive components of the lymph node such as vascular or stromal material are often admixed with the cells of interest, and appropriate gating is needed to eliminate such cells.
3. *Scanty cells may be missed*: It is difficult to identify Reed-Sternberg cell in the specimen of Hodgkin's lymphoma because of scanty R-S cells in the specimen.
4. *No light chain restriction*: All cases of B-NHL may not always show light chain restriction. This may be due to failed expression of light chain on the surface of the cell.
5. *High cost of FCM instrument*: FCM is overall a costly technique and needs good skill.

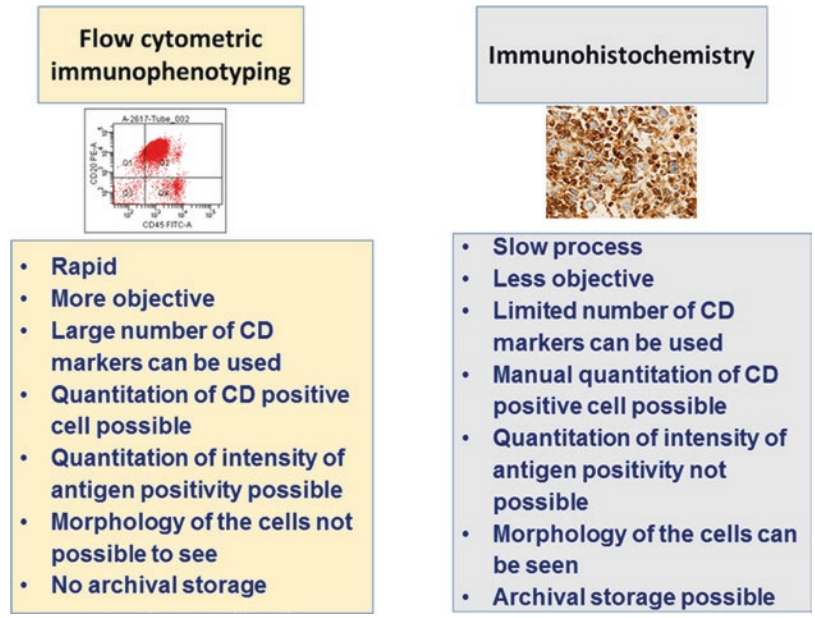

Fig. 17.10 The diagram highlights the differences between flow cytometric immunostaining versus immunocytochemistry

17.7.2 Flow Cytometry Features of Different Lymphomas

Table 17.4 highlights the different CD markers of lymphoma cases.

17.7.3 Apoptosis [14, 15]

FCM is one of the important technologies to detect apoptotic cell death. Quantitation of the apoptotic cells is possible with the help of light scatter (a loss in forward light scatter), plasma membrane changes, and DNA content (a sub-diploid peak).

17.7.4 Assessment of Sub-G1 Fraction of Apoptotic Cells

1. Cells are washed in 1500 RPM for 5 min in PBS.
2. Resuspend the cells in PBS and keep the concentration as 1.5×10^6 cells/ml.
3. Take the cells in 60 µL of PBS.
4. Add 1 ml ice-cold 70% ethyl alcohol drop by drop.
5. Keep for 1–2 h in −20 °C for permeabilization.
6. Wash the cells in PBS by centrifuging.
7. Discard the supernatant fluid.
8. Add 1 ml solution containing propidium iodide, RNAse and Triton X. [RNAse 2 mg, Triton × 10 ml and PI 0.40 ml of (500 µg/ml)]

Table 17.4 CD expression of different lymphomas

Diagnosis	Immunophenotyping
Small lymphocytic	Positive for: CD 5, CD 23, CD 19, CD 20 Co-expression of CD 5 and CD 23 Negative for: CD 10
Mantle cell lymphoma	Positive for: CD 5, CD 19, CD 20 Paraffin section: Cyclin D1, FMC7 Negative for: CD 10, CD 23
Follicular lymphoma	Positive for: Surface Ig, CD 10, CD 19, CD 20, Bcl2, Bcl6 Negative for: CD 5
Lymphoplasmacytic lymphoma	Positive: Cytoplasmic immunoglobulin, CO I9, CD 20, CD 22, CD 79a, CD 38 Negative: CD 5, CD 23, CD 10
Marginal zone lymphoma	Positive: Surface Ig, CD 19, CD 20 Negative: CD 5, CD 10, CD 23
Hairy cell leukaemia	Positive: CD 103, CD 11c+, CD 25+ Negative: CD 10, CD 3, CD 5
Plasmacytoma/multiple myeloma	Positive: CD 38+, CD 138+ Negative: CD 10, CD 3, CD 5, CD 23
Precursor B lymphoblastic	Positive: Tdt, CD 10, CD 19, CD 20 Negative: Surface Ig
Burkitt's lymphoma	Positive: CD 10, CD 19, CD 20, surface Ig+ Negative: CD 5, CD 23 Ki67 index is more than 85%
Diffuse large B cell lymphoma	Positive: Surface Ig+/−, CD 10+/−, CD 19, 20 Negative: CD 5 (90%) Ki 67 index less than 90%
T lymphoblastic	Positive: TdT, CD 3, CD 7, CD 4+/−, CD 8
Peripheral T cell	Positive: CD 3, CD 7 and CD 4/CD 8
Anaplastic large cell	Positive: CD 30 (Ki-1), EMA, ALK Negative: CD 45+/−, CD 3+/−

9. Incubate for 1 h in the dark.
10. Vortex and then run in FCM.
11. Measure the sub-diploid population of cells.

17.7.5 Apoptosis Detection by Annexin V Assay

1. Wash the cells in PBS by centrifuging.
2. Discard supernatant fluid.
3. Keep the cells and keep concentration of 1.5×10^6 cells/ml.
4. Resuspend cells in 500 µL of binding buffer.
5. Add 5 µL of annexin V conjugated with FITC.
6. Incubate at room temperature for 5 min in the dark.
7. Run in FCM for the detection of annexin-binding cells.

FCM in HIV Infection The quantitation of CD 4 and CD 8 count in the blood sample of HIV-infected patients is possible by FCM. This is very helpful in monitoring the HIV patients.

Reticulocyte Count FCM analysis of reticulocyte is a standard and preferred method. Auramine O and thiazole orange are FDA-proved reagents for reticulocyte analysis using FCM.

Steps [16]

1. Thiazole orange reagent (1 ml).
2. Add 5 ml peripheral blood.
3. Incubate for 30 min.
4. Vortexing the mixture.
5. Run in FCM.

Predicting Response to Monoclonal Therapy *As a therapy of low-grade NHL*, rituximab, a monoclonal antibody, is applied against CD 20 on B cells. The follow-up of these cases needs the close monitoring of the measurement of monoclonal antibody CD 20. This is possible by the help of FCM [17].

Detection of Minimal Residual Disease
Multicolour FCM by using a panel of antibody against a wide range of CD markers is helpful in rapid detection of minimal residual disease (MRD). A panel consisting of CD 19/CD 34/CD 10/TdT or CD 19/CD 34/CD 10/CD 13 is used in B cell NHL. Aberrant expression of markers indicates MRD. In case of T cell NHL, CD 34/CD 3 panel is used [18].

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Digital Image Analysis and Virtual Microscopy in Pathology

18

18.1 Introduction

In the last few decades, there is massive development of the computer technology followed by marked advancement in the field of high-resolution image digitization, image storage, extraction of features and analysis. Virtual microscopy has also significantly advanced in the last few years. Previously the pathologists used to give opinion on holistic viewing of the image of the tissue/cells. Presently there is increasing demand to provide semi-quantitative features along with the quantitative information of various biomarkers on the tissue section or cytology smear [1]. Digital image analysis (DIA) provides more objective and consistent information of the images and also helps in the diagnosis, grading and classification of the disease. It provides various prognostic information and guidance to therapy. Nowadays it is essential to have a good knowledge on digital analysis and virtual microscopy for the effective improvement of reporting tissue specimen.

Applications of Digital Pathology The digital pathology has the following applications in the field of pathology (Box 18.1):

1. *Education*: Presently the entire slides can be scanned and stored in the computer system. The slides can be available in the web page or in the parent computer. The classical cases can be used for teaching purposes [2].
2. *Online opinion (telepathology)*: With the help of digital imaging system, it is possible to send the scanned image of the whole slide to the distant centre. The experts can examine the virtual slide and give their opinion. Thereby the physical transfer of the slides can be avoided, and rapid opinion may be available in a particular case [3].
3. *Detection of malignancy*: DIA is useful in the detection of malignant cells in cytology smears and in images of histopathology sections [4, 5]. It helps in the detection of false negative cervical cytology smears. Large number of slides can be re-screened after initial manual screening. In case of any suspicious cells, the computer gives alarm signal, and the slide is again screened in that particular area to avoid any false negative report.
4. *Detailed morphologic study of DNA and chromosomes*: DNA and structure of chromosome have been studied with the help of digital image analysis [6].
5. *Pattern recognition and grading of carcinoma*: Image analysis has been applied to diagnose and grading of carcinomas. Accurate grading of prostate and breast carcinoma were done by different workers on the basis of image analysis [7, 8].

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Box 18.1: Applications of Digital Image Analysis

- *Education*: The digital slides to use for teaching.
- *Telepathology*: Digital slides to use for immediate online opinion.
- *Detection of malignancy*: Malignant cells are detected such as in cervical smear.
- *Detailed morphologic study* of DNA and chromosomes.
- *Pattern recognition and grading* of carcinoma.
- *Quantitation of immunohistochemistry.*
- *Assessment of aggressiveness* of a tumor for personalized medicine.

6. *Quantitation of immunohistochemistry*: With the help of digital image analyser and appropriate software, it is now possible to quantify the HER 2/ER/PR immunostaining on the histopathology section. Automated quantification of fluorescent in situ hybridization (FISH) is also possible on histology tissue [9].
7. *Assessment of aggressiveness of a tumor for personalized medicine*: With the help of digital image analysis, it is now possible to find out the aggressiveness of individual tumor in individual persons. It is often seen that the same tumor with same type of treatment has different outcome in two different patients. Digital image analysis can provide useful information on (a) tumor morphology, (b) tumor classification, (c) tumor grading, (d) tumor stroma reaction, (e) quantification of biomarkers and (f) molecular phenotypes. The combined data of all those features may be helpful to plan for personalize treatment [10].

18.2 Basic Principles of Image Analysis

The basic principles and steps of image cytometry includes below (Fig. 18.1):

- *Image digitalization*: Pixel is the basic unit of any image. Each pixel is located in particular X and Y axis. On the basis of the grey intensity of the pixel, the individual pixels are given a particular grey value. In a coloured image in a particular pixel, three values are given based on the intensity of red, green and blue colour. Therefore the pixels are stored in the computer as the representation of a particular grey value. This is known as digitization of the image. From the digitized stored image, the original image can be easily retrieved.
- *Image detection and segmentation*: By adjusting grey threshold value, one can detect the cells or image of the interest.
- *Image editing*: With the help of editing, the images are processed, and the unwanted objects are eliminated from the background.
- *Feature extraction*: Next important step of image analysis is the measurement of various geometric data from the object of interest that include diameter, perimeter, area, roundness and texture. The other components of the features are the margin or periphery of the duct, gland or cell-stromal interface detection.

18.3 Details of the Image Analysis Steps

(a) *Image processing*: In case of image processing, the images are modified to have better quality. We use three techniques for the restoration of the image: (1) image smoothing, (2) image denoising and (3) image enhancement.

Image smoothing: Gaussian filtering or bilateral filtering is used to remove the background noise of the image and also to remove finer details.

Image denoising: The noises that are produced at the time of image acquisition and reconstruction should be removed by image denoising technique. Wavelength thresholding, variational methods or robust statistic is used for image denoising [11].

Image enhancement: Image enhancement technique is used to enhance the contrast

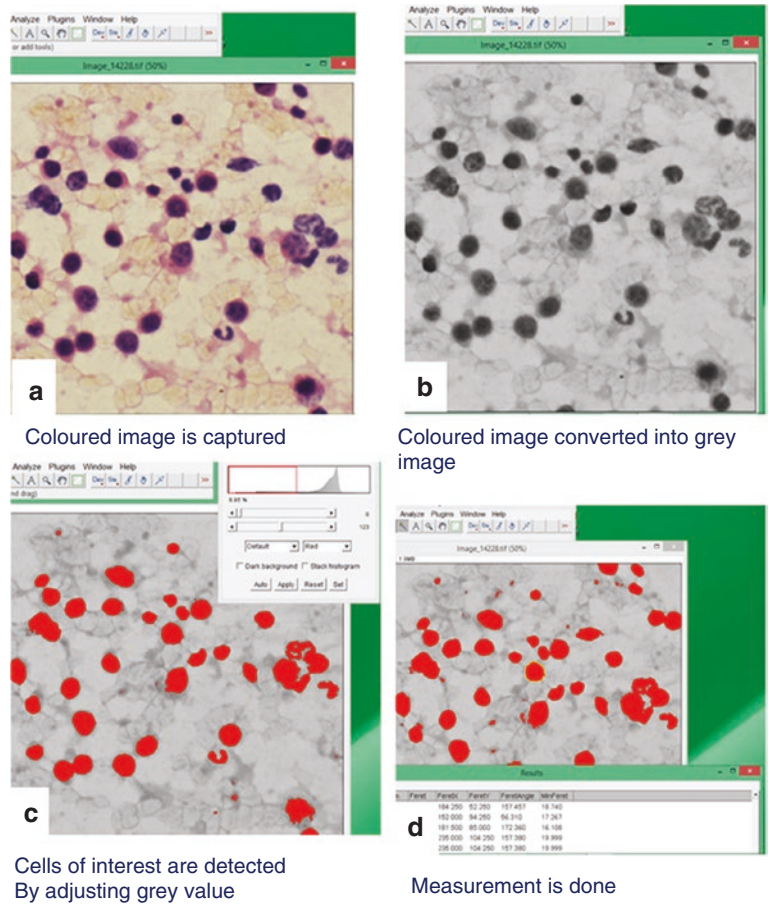

Fig. 18.1 Illustrated figure of the basic steps of image analysis: (**a**) at first the coloured image is captured. (**b**) Subsequently the coloured image is converted into grey image. (**c**) The cells of interest are detected by adjusting the grey value. (**d**) Finally the measurement is done

between the objects of interest and the background. We use adaptive filters or inverse anisotropic diffusions technique for image enhancement.

(b) *Automated segmentation*: Automated segmentation is the most important step to have a successful analysis of the digital images. In fact, this is the most challenging area of the digital image analysis. Automated segmentation can be done by:

- *Grey level threshold method*: As the nuclei are differently stained, so they can be separated by adjusting the grey threshold [12]. At first, the colour of the different components of the image is noted such as nuclei, cytoplasm and stromal area. Then the image of the interest is segmented by utilizing the colour and gradient information [13].
- *Similarity of the region of image*: The image is segmented according to the similarity and dissimilarity of the data. At first scale-space filtering technique is used followed by watershed clustering technique [14].
  - Scale-space filtering: In this technique the filtering is done in a continuum of scales by applying Gaussian filters.
  - Watershed clustering technique**:** This is also known as region-based segmentation approach. The watershed lines are constructed around the boundary of the different objects of interest, and this watershed technique is applied to determine the individual objects in the image. The major problem of watershed technique is over-segmentation and thick watershed.

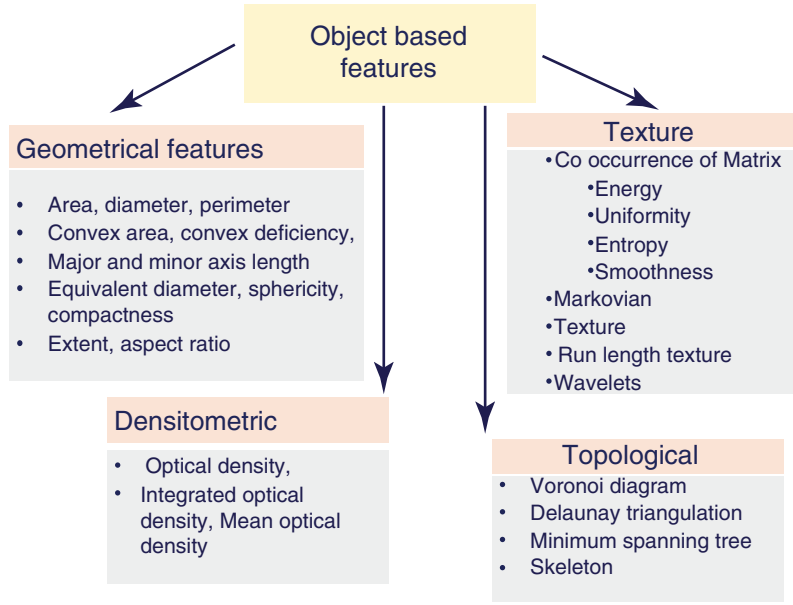

Fig. 18.2 Object-based features of digital image are highlighted here. There are four types of object-based features: (1) geometric features, (2) textural features, (3) densitometric features and (4) topological features

(c) *Feature extraction and unnecessary data reduction*: The different sorts of features are extracted from the digital images such as (Fig. 18.2):
   1. *Geometric features*: Shape and size
   2. *Topological features*: Voronoi diagrams, Delaunay triangulation and minimum spanning trees
   3. *Densitometric study*: Optical density, integrated optical density and mean optical density
   4. *Textural features*: Co-occurrence of matrix (energy, uniformity, entropy, smoothness), Markovian texture, run length texture, wavelets and fractal dimension

It is essential to reduce the vast quantity of data for the feasible interpretation. Either linear or non-linear data reduction techniques can be used. The linear techniques of data reduction include:

- Principal component analysis
- Multidimensional scaling
- Linear discriminant analysis

The non-linear data reduction techniques are:

- Spectral clustering
- Isometric mapping
- Locally linear embedding
- Laplacian eigenmaps

After significant data reduction, the remaining features can be used as input features to determine the disease identification, classification or grading.

Instruments Requirements Nowadays no special equipments are needed for image analysis. A digital camera attached to the microscope and a suitable software is sufficient for image analysis. ImageJ software is now freely available in the Internet (https://imagej.nih.gov/ij/download.html), and various geometric measurements are possible by this software. There are many other free softwares available in the Internet to do image analysis.

18.4 Morphologic Features

The various morphometric features can be detected with the help of digital image analysis such as (1) geometrical shape and size, (2) densitometric features and texture, (3) pattern of distribution and (4) chromatin specific.

Geometrical Shape and Size These features include cell diameter, perimeter, area, cell shape, nucleo-cytoplasmic ratio, etc. Area is calculated by measuring the number of pixels occupied by the cell. Similarly the maximum number of pixels in the particular axis represents the maximum diameter of the cell (considering that the cell is round). Eccentricity is calculated as dividing the minor axis length by major axis length. The nuclear shape is described by circularity, eccentricity and irregularity of the nuclei. These shape features are important to identify malignant cells.

Texture The texture analysis of the image of interest gives important visual clues. Nuclear texture analysis helps in the assessment of interchromosomal coarseness. The most commonly used statistical method of texture analysis is grey level co-occurrence of matrix (GLCM). The following parameters describe GLCM:

- Energy
- Uniformity
- Entropy
- Smoothness

Densitometric Features The various densitometric features include minimum grey value, maximum grey value, average grey value, mean grey intensity, standard deviation, kurtosis and the span of histogram.

Pattern Recognition There are significant advances in image analysis as whole slide scanning is available along with fluorescent images data. The spatial arrangement of the objects is the important step of the pattern recognition.

The following features are used for the spatial arrangement of histopathology image analysis:

1. The local information around a single cell cluster
2. The global information around a single cell cluster
3. Information regarding the global connectivity of the graph
4. Information from the spectral graph

Data Fusion The image analysis data can be combined with the epigenetic, genetic and proteomic data. These complex data sets representing the spatial arrangement of the histopathology images, cell morphological features and genetic/epigenetic information may be very helpful in the disease classification, the grading of the tumor and the recognition of the aggressive subset of the disease [15] (Fig. 18.3).

Subcellular Quantification of the Substances The computer-assisted digital image analysis is able to quantify the immunohistochemically or immunoflurescently stained cellular target proteins on the slide or in tissue microarray specimen [16]. With the help of using colour code, size and shape of the positively stained nuclei, the digitally scanned slide can be quantified for particular substances. Various algorithms are used to quantify the target proteins such as HER 2 receptors, oestrogen and progesterone receptor (ER/PR), etc. Various commercially produced softwares including hardwares are now available to quantify the cytoplasmic, nuclear and membranous staining of the protein [9]. Automated detection of fluorescent in situ hybridization (FISH) is also possible with the help of computer-assisted image analysis technology [9].

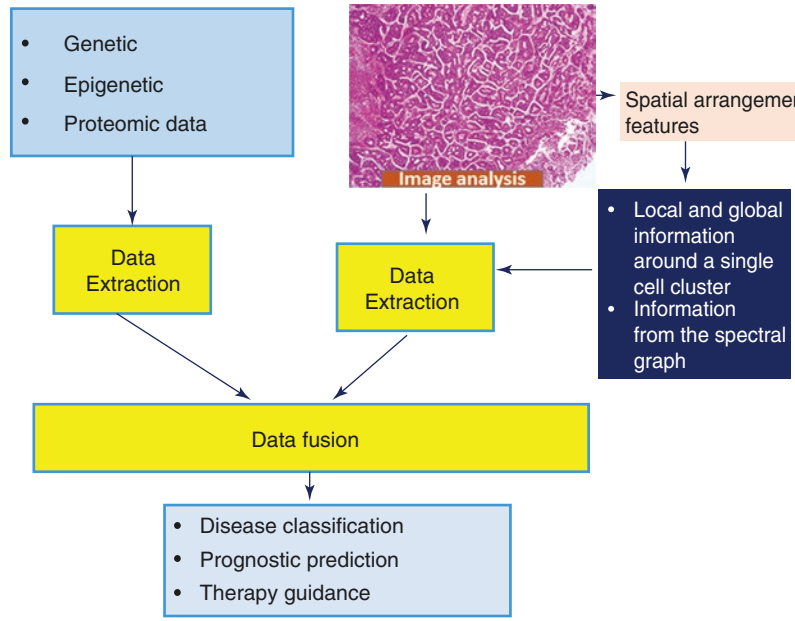

Fig. 18.3 Data from digital images can be fused with genetic, epigenetic and proteomic data for better classification, therapeutic guidelines and prognostic assessment

18.5 The Current Problems of Digital Image Analysis

The various problems in DIA are described below (Box 18.2):

- *Auto-segmentation*: As mentioned before till now, auto-segmentation unaided by any human interference is the major challenge in digital image analysis. The digital analysis system should be capable to identify the images of interest. It should also process the image and finally extract the data from the images. Different modules have been used to solve the problem such as edge-based, region-based and model-based module.
- *Decision on individual patient*: It is very important to take the decision of diagnosis or classification or grading, etc. in individual patients. Simple linear statistics is not helpful to take decision on individual patients. Different knowledge-based techniques have been used to solve this problem. The commonly used knowledge-based techniques are (1) K-means and spectral clustering, (2) artificial neural network, and (3) supervector machine.

Box 18.2: Limitations and Difficulties of Image Analysis

- *Auto-segmentation*: Various modules used such as edge-based, region-based and model-based.
- *Decision on individual patient*: Knowledge-based software helps in this aspect.
- *Three-dimensional imaging data*.
- *Computational complexity and immediate application*: Complex data set and difficult to handle.

- *Three-dimensional imaging data*: We gather two-dimensional image data which is too some extent not the real representation of the cell. Three-dimensional construction of the image and analysis of the data are the most informative. With the help of the 3D reconstruction, the ultrastructure of the cells is also better studied [17].
- *Computational complexity and immediate application*: The large amount of data generated by the image analyser gives significant computational complexity and this may

interfere the immediate application of the system.

18.6 Virtual Slide and Web-Based Teaching

Till now training and education of the histopathology and cytology are based on the glass slides and traditional microscopy, text book and web pictures. In the last few years, there is massive advancement in the digital technology, and presently the entire slide is available in the computer as "whole slide imaging" with the help of whole slide scanning. In this whole slide imaging, a complete digital slide is generated. The observer can examine any part of the slide by increasing or decreasing the magnification. The particular area of the section is zoomed immediately like the glass slide under the microscope [18–20].

18.6.1 Advantage of Virtual Slides

There are several advantages of virtual slides (VSs) over conventional light microscopy (CLM). These advantages are mentioned in Box 18.3.

The VSs have revolutionized the area of conventional concept of glass slides and microscopy. Unlike conventional microscopy VS needs only a computer with Internet access. Therefore there is no need of multiple microscopes or multiheaded microscopes for teaching and training. The greatest advantage of VS is that simultaneously many people may have the access to the slides and therefore it can be used in teaching or training in pathology (Box 18.3). Same image is seen by both the students and teachers, and therefore there is better interaction in teaching session. The initial low-powered image of the slide provides the orientation of the tissue. The annotation on the image also highlights the important features of the slide for better understating of the lesion. Moreover, the VSs may be accompanied with immunocytochemistry or other histochemical stains that help in the proper diagnosis and classification of the lesion. In addition, clinical history, simple radiographs, computed tomography or magnetic resonance imaging can be added along with the VSs. Therefore a complete disease picture may be available along with VSs.

Box 18.3: Advantages of Virtual Slides

- Multiple users can view the slides.
- Only a computer and Internet access are enough to teach.
- Same images are seen both by students and the teacher so better interaction is possible.
- No need to have multiple microscopes and their maintenance.
- Consistent classical cases can be stored as VSs
- More than one slide can be viewed in a same screen to compare the lesion.
- The specific area or the particular object can be labelled for attention and display.
- No fading or damage of digital slides.
- No need of multiple slides and thereby tissue is not exhausted.
- The cases can be accessed instantly and there is no need of museum staff.
- Distant teaching (tele-education) and even self-learning.
- Virtual workshop.

Distant learning is possible with the help of VSs. One can even conduct online workshop as the VSs can also be available in Internet site. Moreover, the good quality VS of the classical disease can be used for conducting examination. Unlike conventional glass slides, VSs will never fade and there is no need of extra slides, so tissue will not be exhausted particularly in case of small biopsy specimen.

18.6.2 Disadvantages of Virtual Slides

One of the major disadvantages of the VSs is the initial cost of the equipments [18] (Box 18.4). The slide scanner and the accessories of the

Box 18.4: Disadvantages of Virtual Slides

- Initial high cost of the equipments
- Need of huge storage space
- Unfamiliarity to handle the conventional light microscope

whole slide scanning system are very costly. The next important problem of VS is the storage of the high-resolution data. Huge amount of disc space is required for the storage of the individual slides. Therefore servers with high capacity storage capability are needed.

The other probable disadvantage is the reduced interaction of the students and light microscopic examination of the slides. This is not a problem of the undergraduate training as very few students get a chance to handle light microscope in their future career. However, for the postgraduate training, overemphasis of training through the VSs may be detrimental to the routine examination of the independent diagnosis of the glass slide.

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19 Liquid-Based Cytology and Automated Screening Devices in Cytology Sample

19.1 Introduction

The liquid-based cytology (LBC) is an increasingly popular technique of the preparation of the cervical sample and also other exfoliative samples such as effusion fluid, sputum, bronchoalveolar lavage, etc. This technique is relatively costly than the other sample collection and preparation. However, LBC preparation provides clean, monolayered smear in small area of the slide. As the smear is free of blood, mucus and drying artefact, so it is easy to interpret [1, 2].

19.1.1 Advantages of LBC Over Conventional Smear

The major advantages of LBC over conventional preparation (CP) include:

- Majority of the collected cells are available in the liquid medium of the collection vial, whereas the major part of the collected cells is sticked in the spatula of the convention smear preparation and the cells are thrown in the waste basket.
- LBC preparation is completely free of any air-drying artefact.
- There is almost complete absence of any blood, mucus or necrotic debris in LBC preparation, and the cells are present in small area which is easy to screen (Fig. 19.1).
- Monolayered preparation of the cells is present in certain LBC preparation.
- HPV test is possible for the residual material of LBC sample.
- The monolayered cell preparation may be useful in automated detection of malignant cells in the smear.

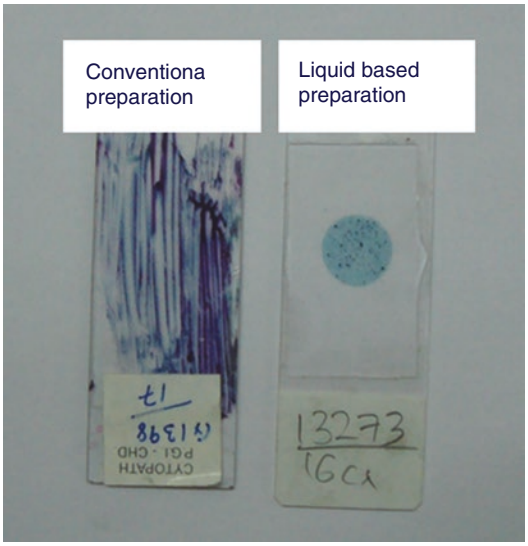

Fig. 19.1 Smear of conventional and liquid-based cytology. Note the small area of liquid-based smear

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Box 19.1: Advantages of Liquid-Based Cytology

- No air-drying.
- Representative cells in adequate number.
- Small smear area is easy to screen.
- Clean background.
- Monolayered cells.
- HPV testing.
- Multiple slides can be made.

19.1.2 Limitations of Liquid-Based Cytology

The major limitations of LBC are the initial cost of the machine. Moreover the collection fluid, spatula and processing cost are also significantly high than CP (Box 19.2). The laboratory staffs also need adequate training for the preparation and screening of the smear. The cells are dispersed from the neighbouring cells, and certain architectural features in adenocarcinoma may be lost [3]. Moreover the smear may be free of any tumor diathesis, and therefore the diagnosis of squamous cell carcinoma may be difficult. There is no definite evidence that LBC reduces the inadequate smear rate or it detects more number of high-grade squamous intraepithelial lesion (HSIL) [4].

Collection Procedure of LBC

- Patient should be in lithotomy position.
- Inspect the cervix with the help of a speculum.
- Clean any watery discharge or mucus by wet cotton.
- Insert the cervical broom within the cervical canal so that shorter bristles touch the ectocervix.
- Rotate the broom several times in clockwise and anticlockwise direction.
- Remove the broom and rinse it vigorously within the liquid provided by the company.
- Cap the vial tightly for further processing.

Box 19.2: Limitations of Liquid-Based Cytology

- High cost
- Trained cytotechnicians needed
- Loss of certain background information
- No define evidence of increased sensitivity of the detection of HSIL cases

19.2 Sample Processing

Presently there are two commercially available liquid-based technologies that are approved by Food and Drug Administration (FDA), USA: ThinPrep Pap test and SurePath test. The preparation techniques of these two techniques are different and are described below.

19.2.1 ThinPrep (Cytic, UK) (Fig. 19.2)

The steps of ThinPrep processing are:

Dispersion and collection of the cells on the filter

- Collect the sample in PreservCyt fixative solution supplied by the company.
- The cylindrical tube with attached filter in the lower surface is submerged within the vial containing the cells in the PreservCyt solution.
- The cylinder rapidly rotates within the vial and disperses the cells mechanically.
- Simultaneously negative suction pressure is applied within the cylinder that drains the fluid of the vial.
- The cells cannot pass through the filter and sticks on the undersurface of the filter of the cylinder tube. The negative suction pressure can be adjusted by an inbuilt software to control the flow of the fluid.

Cell Transfer The trapped cells are now accumulated on the surface of the filter attached with the cylindrical tube. The machine now

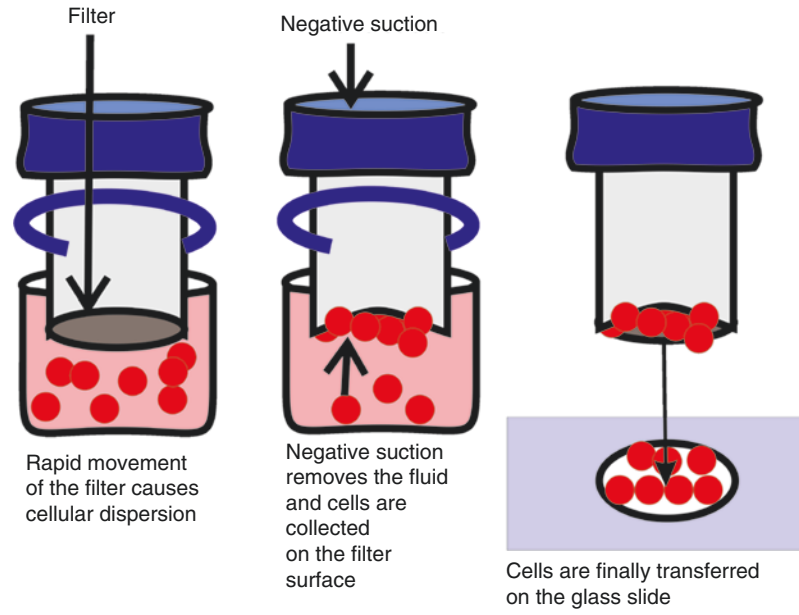

Fig. 19.2 Schematic diagram highlighting the basic principle of ThinPrep technique. The rapid movement of the filter causes cellular dispersion. Simultaneously negative suction is applied within the vial of filter that helps to remove the fluid and the cell sticks on the filter

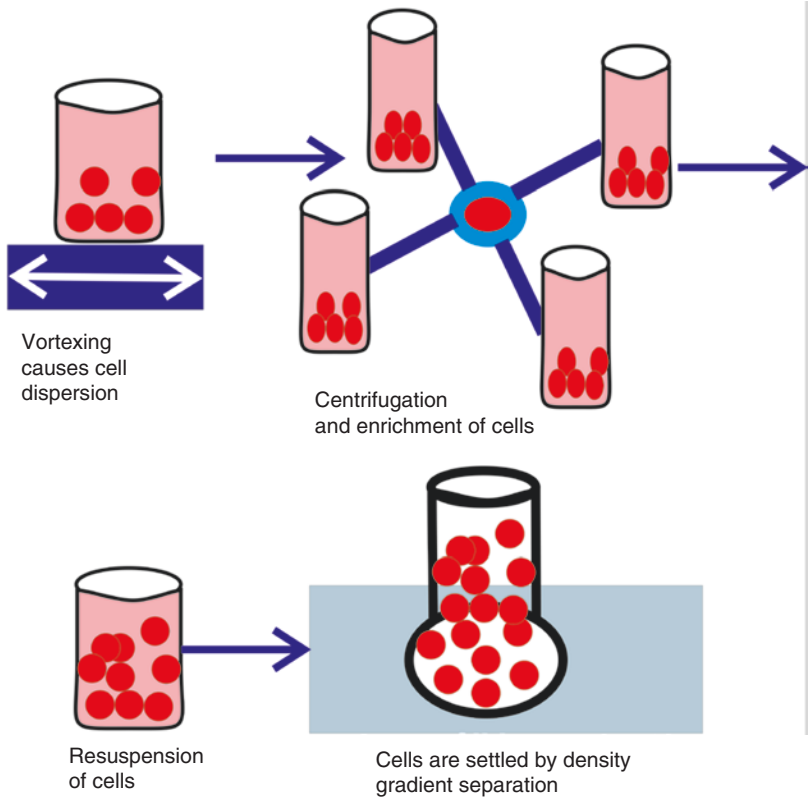

Fig. 19.3 Schematic diagram highlighting the basic principle of SurePath technique. The cells are dispersed by vortexing. Cell-rich suspension is made by buffy coat preparation. These cells are taken out and resuspended in fluid. Finally the cells are settled down by gravity on the glass slide

automatically transfers the cells from the surface of the vial to the glass slide. The glass slide is automatically submerged in the fixative solution.

19.2.2 SurePath Test (Fig. 19.3)

The overall steps of SurePath test are highlighted in Fig. 19.3.

Fig. 19.4 Vortexing causes cell dispersion in SurePath technique

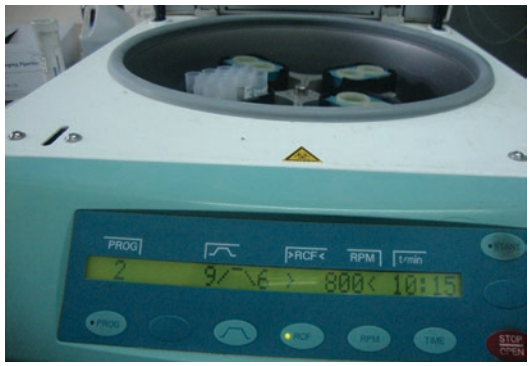

Fig. 19.5 Centrifugation of the sample with the addition of density gradient mixture helps to enrich the cells

Vortexing (Fig. 19.4) At first with the help of vortexing, the cells are mechanically dispersed.

Cell enrichment

- Mix the sample with PrepStain® Density Reagent and centrifuge (Fig. 19.5).
- Decant the supernatant elements containing blood, mucus and necrotic debris by applying vacuum suction.
- Recentrifuge the remaining fluid to have a cell-rich pellet.

Resuspension
Re-vortex the cell-rich pellet

- Resuspend the cells.

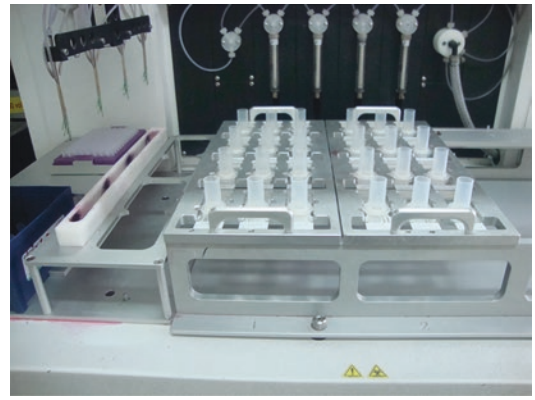

Fig. 19.6 The vials are kept in the settling chamber to settle the cells by gravity

- Transfer the solution in the PrepStain slide processor.

Cell sedimentation

- Pour the cell suspension in the PrepStain® Settling Chamber (Fig. 19.6).
- With the help of gravitational force, the cells are sedimented on the pre-coated slide.

19.3 Comparison of These Two Techniques

The processing techniques of the two above-mentioned systems are completely different. ThinPrep technique is more automated than SurePath technique. Both the techniques have certain advantages and limitations. Table 19.1 highlights the comparison of these two techniques.

19.4 Automated Screening Devices

There is no fully automated screening device in the market. We only have semiautomated systems. There are two FDA-approved semiautomated screening devices in the market:

1. BD FocalPoint GS Imaging System
2. Hologic ThinPrep Imaging System

Table 19.1 Comparison of Pap test and SurePath techniques

ThinPrep	SurePath
Methanol-based collection fluid	Ethanol-based collection fluid
Completely automated	Manual
Cell dispersion is done by circular rotatory filter submerged within the vial	Cell dispersion is done by manual vortexing
Cells are concentrated on the surface of the vial by negative suction	Cells are concentrated by density gradient, and therefore the cells may be present in more than one layer
Blood and mucus may block the pores of the filter	Better cellularity as there is such problem
Good monolayered cell preparation possible	Cells are in multiple layers

19.4.1 BD FocalPoint GS Imaging System [5, 6]

FDA has approved these screening devices for both primary and rescreening of SurePath and conventional smear.

The BD FocalPoint GS Imaging System (FPGS) has two parts:

1. BD FocalPoint™ Slide Profiler
2. BD FocalPoint GS Review Station

BD FocalPoint™ Slide Profiler The slide profiler scan the cervical smear and analyse the specific cytological features. The system finally classifies the slide as "no further review" or "review" category.

- Label the cervical smear slide by the specific barcode, and load it into the BD FocalPoint™ Slide Profiler.
- There are total 36 trays and each tray contains 8 slides.
- Each slide automatically moves from the tray to the microscopic stage.
- The device reads the barcode and verifies the physical integrity of the slide.
- The system then scans the slides both in low and high power.
- The slides are ranked according to the degree of abnormality, and the device gives a score to each slide depending on the probability of abnormality.
- The slide profiler classifies the slide as:
  - No further review: Highest probability that the smears are normal.
  - Further review: Here manual review of the slide is needed to confirm the abnormality. Usually 75% of such cases contain abnormal cells.

BD FocalPoint GS Review Station This is a review station and the cytotechnologists followed by cytologist review all the slides to detect the abnormality.

- The slides are placed over the printed PAPMAP that indicates the maximum possibility of the microscopic field of view (FOV) containing the abnormal cell.
- Screen the areas of FOV.
- Reconfirm the findings of the cytotechnologist.

19.4.2 Hologic ThinPrep Imaging System [7, 8]

Hologic ThinPrep Imaging System has two parts:

1. ThinPrep Imaging System
2. ThinPrep Review System

ThinPrep Image Processor The ThinPrep image processor (TIP) system scans the slide, detects the abnormal cells and marks the field of view for rescreening. The system analyses the various cellular features along with the optical density of the nucleus. Therefore the smears have to be stained in ThinPrep 2000 or 3000 Processor with ThinPrep Stain. This particular processing and stain is needed to maintain the consistency of the stain. The integrated optical density of the nucleus is measured from the Feulgen stained nuclei.

Table 19.2 Differences of BD FocalPoint GS Imaging System versus Hologic ThinPrep Imaging System

BD FocalPoint GS Imaging System	Hologic ThinPrep Imaging System
Special slide preparation is not mandatory	It is mandatory to have ThinPrep processing and stain
The cellular features mainly nuclear size, shape, nucleocytoplasmic ratio, etc. are picked up and analysed	The cellular features along with optical density of the nuclei are analysed
The slides are ranked according to "device score" and labelled as "review" or "no review"	The most abnormal 22 fields are recorded in each slide. No ranks to the slides are given
Cytotechnologist can screen only "review" labelled slides	Cytotechnologist has to screen every slides again in those 22 marked areas

- Place the slides in the cartridge that contains 25 slides. The total of 10 such cartridges can be operated at a single time.
- The slides within the cartridge are imaged.
- The system records 22 FOV of the microscopic fields that may contain abnormal cells.
- It transforms the information to the review scope

Review Scope Place the slide in the review scope.

- Access the stored location of the 22 FOV by the review scope.
- Now the areas are screened as geographical manner rather than rank of abnormality.
- If any abnormal cells are detected, then screen the entire slides.

The difference of operating systems of the two technologies is highlighted in Table 19.2.

19.4.3 Comparison of Manual and Automated Devices

In a large study these two automated systems were compared to the manual screening [9]. It was noted that the automated screening devices are 8% less sensitive than manual screening.

Table 19.3 Comparison of the automated screening versus manual screening

Automated screening	Manual screening
No chance of fatigue	Tiring and boring job
Automated system follows consistent logic	Erratic and subjective approach. Inexperienced worker may miss the abnormal cells
Machine takes fixed amount of time	There may be variable time period of screening and technologist takes longer time
Very costly device	Cheap

The study group raised doubts about the implementation of the automated screening devices because of the reduced sensitivity and cost-effectiveness. There is no doubt that automated screening devices increase the productivity of the cytotechnologists as less number of fields are needed to screen by them. Automation may reduce the load of the tedious job of the cytotechnologists (Table 19.3). However, the increased economic burden in the screening program may be one of the major obstacles of the automation. Moreover, the HPV testing along with manual screening may give more meaningful result. Therefore, in the future we may have to give a serious thought before we implement automated screening.

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20 Polymerase Chain Reaction: Principle, Technique and Applications in Pathology

20.1 Introduction

Polymerase chain reaction (PCR) is one of the most important techniques in molecular pathology [1, 2]. With the help of PCR, the single or the pieces of target DNA can be amplified many folds. This technique is now an integral part in every modern laboratory for both the diagnosis and research use.

20.1.1 What Is PCR and How It Works?

PCR acts like a "molecular photocopying" machine and amplifies the specific target DNA. The basic principles of PCR are:

- Double-stranded target DNA is made into single-stranded DNA by applying heat.
- Two oligonucleotide strands or primers are added. The oligonucleotide strand binds with its complimentary DNA strand to the 3′ ends.
- The DNA strand is now extended with the help of DNA polymerase (Taq polymerase). This polymerase enzyme incorporates the nucleotides in the DNA to make it elongated.
- The cycle is repeated.

20.2 Steps of PCR

The basic steps of PCR are described as [1, 2] (Box 20.1, Fig. 20.1):

Box 20.1: Principle of Polymerase Chain Reaction

The specific gene is amplified by using a pair of DNA primer, heat-resistant DNA polymerase enzyme and nucleotides.

Steps

- *Denaturation*: Heat breaks the double-stranded DNA to single-stranded DNA.
- *Annealing*: Forward and reverse DNA primer bind with the complementary DNA strand in 3′ region of each separated DNA strand.
- *Extension*: Heat-resistant DNA polymerase (Taq polymerase) enzyme grabs the nucleotides and extends the DNA strand from 3′ to 5′ direction.

Repetition of this thermal cycle for 25–30 times increases the DNA product.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_20

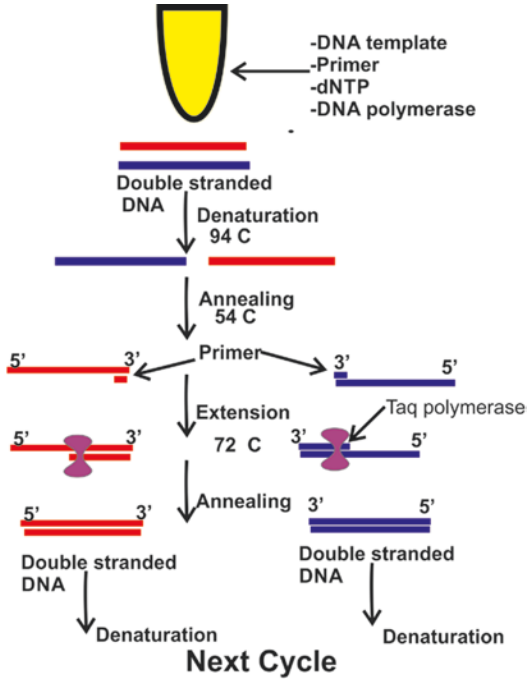

Fig. 20.1 Schematic diagram shows the basic principle of PCR. The basic steps are denaturing of double-stranded DNA to single-stranded DNA, annealing with the primer and extension of DNA strand

- *Denaturation step, 94 °C*: DNA is heated to 94 °C to make it single stranded. Only 1–2 min are given to this heating process in each cycle.
- *Annealing, 54 °C*: The temperature is rapidly cooled. In this lowered temperature, the primer quickly anneals with the respective site of DNA. With the help of Taq polymerase, the reaction starts at the primer-DNA template site.
- *Extension, 72 °C*.
- The complementary nucleotides are attached from the 3′ to 5′ end of DNA. There is an exponential increment in the number of genes in each cycle. At least 30 cycles of denaturation-annealing-extension is done in each PCR.

20.2.1 Essential Ingredients of PCR

- *Primers*: The primers are the small pieces of artificially made DNA strands that are actually the complementary strands of the 3′ end of each strand of target DNA. Primers usually consist of 20–30 nucleotides.
- *DNA polymerase (Taq polymerase)*: Taq polymerase is a type of DNA polymerase enzyme that extends the new DNA strand. It combines at the end of the primer and then sequentially adds new nucleotides to the DNA strand at 3′ end complementary to the target DNA. High temperature (94 °C) is needed to separate the double-stranded DNA. Ordinary DNA polymerase breaks down in this temperature. However, the Taq polymerase has the unique characteristic to work efficiently in higher temperature. This Taq DNA polymerase is extracted from the bacteria *Thermus aquaticus*. These bacteria live in hot spring and can survive there.
- *Deoxynucleotide triphosphates (dNTPs)*: Deoxynucleotide triphosphates (dNTPs) are dATP, dCTP, dGTP, and dTTP. These are the raw material or the basic building blocks of the new DNA strands. The Taq polymerase captures these dNTPs from the working solution and attach them to the terminal part of the primers to extend the DNA chain.
- *The target DNA from the sample*: The target DNA is extracted from the sample.
- *Buffer solution*: It provides the optimum chemical environment for the reaction to occur.
- *Magnesium chloride ($MgCl_2$)*: Magnesium chloride works as a cofactor of the Taq polymerase enzyme.

20.3 Procedure Proper [3, 4]

20.3.1 Basic Precautions

- Always wear gloves to avoid any contamination.
- Completely thaw all the reagents before doing PCR.
- Keep all the reagents in an ice basket throughout the time of PCR experiment.

20.3.2 Equipment

- Thermal cycler
- PCR tubes and caps
- Ethanol-resistant marker
- Micropipettes set

Addition of the Ingredients in a 50 µgredieTube

- **Target DNA**: 1 µLDNA template (1 ng total amount)
- **Forward primer**: 1 µL of 50 µM primer (final concentration is 1 µM)
- **Reverse primer**: 1 µL of 50 µM primer (final concentration is 1 µM)
- **dNTPS**: 4 µL dNTP mix (2.5 mM dNTP, final concentration is 200 µM)
- **Taq DNA polymerase**: 0.25 µL of 5 U/µL of Taq polymerase (total amount 1.25 U)
- **Buffer**: 5 µL 10 X polymerase buffer (commonly this buffer is supplied by the manufacturer)
- **$MgCl_2$**: 5 µL of 25 mM $MgCl_2$

Remember

- Add the template DNA and DNA polymerase just before the PCR to start.
- Please put at least one negative control and if possible one positive control.

20.3.3 Thermal Cycling

- Close the cap of the PCR tubes and then put them in the thermal cycler.

Standard Steps

- **Initial denaturation**: At 94 °C for 1 minute
- **Denaturation**: At 94 °C for 30 seconds
- **Annealing**: 50–60 °C for 30 seconds: The temperature may vary depending on the primer used. The temperature of annealing stage should be 3 to 4 °C lower than the melting point (T_m) of the primers.

$$T_m \text{ of the primers} = \left(4 \times \left[G + C\right]\right) + \left(2 \times \left[A + T\right]\right)^{\circ}\text{C}$$

G = guanine, C = cytosine, A = adenine, T = thiamine

- **Extension**: 72 °C for 1 minute per kilobase of the PCR product
- **Final extension**: 72 °C for 10 minute
- *Termination*: The reaction is terminated by chilling the mixture to 4 °C.

Cycling Time The PCR thermal cycle rapidly heats and cools the PCR reagent mixture. The cycling time depends on (1) size of the DNA template and (2) G-C content of DNA. The number of the thermal cycler is usually set as 25–30 cycles. If the thermal cycle is increased more than 35, then too many unwanted DNA products may be produced.

The product is calculated as

$$M_f = M \times 2^N$$

M_f = final number of DNA molecule, M = initial number of DNA molecule, N = number of PCR cycle

Purification of the Amplified Product
The following measures are taken to purify the PCR products from the reaction solution:

- *Agarose gel electrophoresis of the product*: Agarose gel electrophoresis is done from the portion of the PCR product to verify the validity of the test.
- Note the following things:
  - Any band present in the agarose gel electrophoresis or not?
  - Is there any other bands of different sizes?
  - Is there smear pattern?
  - The successful PCR amplification product shows a single sharp band with expected size.
- *Cloning of products*: In this technique further PCR is done to confirm the PCR product. This is done when the gene is present in very tiny amount.
- *Sequencing of products*: This is done by automated sequencer machine to analyse the sequence of DNA formed as PCR product.

20.3.4 Troubleshooting

The various problems in PCR are described here (Table 20.1):

1. *No amplification of DNA*: The possible causes may be:
   - *Too small amount of DNA template*: If there is very less amount of DNA template, then there may not be adequate amplification. The amount of DNA template should be increased in such condition.
   - *Too stringent reaction condition*: If the reaction condition is kept as very strict, then there may not be any amplification of PCR products.
   - *Reagent is not added*: One or more reagents may not be added in the reaction mixture. The whole reaction should be done again by carefully adding the reagents.
   - *Reagents are not in optimum concentration or expired*: The reagents should be made fresh, and one new reagent is changed at a time to find out which reagent created problem.
   - *Denaturation temperature is either too high or too low*: In such condition change the temperature 1°C low or high at a time.
   - *Primer annealing temperature is very high*: In such case, lower the temperature 2 °C at a time.
   - *Primer dimer formation*: Two primers may self-anneal or anneal with each other. In this case the gel electrophoresis shows a small product of less than 100 base pair. Addition of DMSO may solve this problem. Alternatively hot start thermal cycling may resolve this issue.
   - *Suboptimal number of thermal cycle*: Suboptimal number of thermal cycle may produce less amount of PCR product. In such condition, increase 5–10 more number of thermal cycle.
   - *Faulty primer*: The primer may be faulty and therefore PCR may not function at all. In such case, redesign the primer.
2. *Non-specific product*: Non-specific product may be formed in PCR, and in this case multiple bands with variable lengths are seen in gel electrophoresis. This may be due to:

Table 20.1 Troubleshooting in PCR

Problem	Cause	Solution
No PCR product	Too small amount of DNA template	Increase the amount of DNA template in the reaction mixture
	Too stringent reaction condition	Reduce the stringency
	Suboptimal number of thermal cycle	Increase 5–10 more cycles
	Denaturation temperature is either too high or too low	Change the temperature 1°C low or high at a time
	Primer annealing temperature is very high	Lower the the temperature 2 °C at a time
	Reagent is omitted by mistake	Do the reaction again
	Reagents not in optimum concentration or expired	Make fresh reagents and one at a time in the reaction mixture
	Primer dimer formation	Add dimethylsulfoxide
	Faulty primer	Redesign the primer
	G-C-rich DNA template	Add dimethylsulfoxide
Spurious product	Too less stringency in PCR	Stringent PCR condition to maintain
	Too many number of thermal cycles	Maintain optimum thermal cycles
	Too much DNA template	Reduce the amount of DNA template
	Too many thermal cycles	Reduce the number of thermal cycle 5–10
	Magnesium concentration is very high	Lower the concentration
	Faulty primer	Redesign the primer
	Carry over contamination	Change the place of PCR

- *Too less stringency in PCR*: Too less stringent condition generates unwanted DNA products in PCR.
- *Too much DNA template*: Too much DNA template may produce undesired product in PCR.
- *Too many thermal cycles*: In such case, reduce the number of thermal cycle 5–10.
- *Magnesium concentration is very high*: Adjust the concentration and make it low.
- *Faulty primer*: Redesign the primer.
- *Carry over contamination*: Change the place of PCR.

20.3.5 Enhancing PCR Products Formation

The following measures help to enhance the PCR products [4]:

- *Addition of non-ionic detergents*: Triton X-100, Tween 20, and NP-40 help to stabilize the DNA polymerase enzyme and enhance the reaction. However, these agents may lower the PCR stringency, and undesirable DNA products may be formed. More than 1% concentration of these detergents may have inhibitory effect on PCR.
- *The addition of dimethylsulfoxide (DMSO) in the G-C-rich DNA template (1–2%)*: Addition of DMSO disrupts the base pair and enhances the reaction.
- *Optimized annealing temperature*: It is necessary to optimize the annealing temperature to increase PCR products.

20.4 Types of PCR

There are different types of PCR methods for diagnostic purposes. These are:

1. *Direct PCR*: This is the standard PCR technique as has been described in the previous section.
2. *Reverse transcriptase PCR (RT-PCR)*: In case of RT-PCR, at first cDNA is prepared from RNA of the target sample. This cDNA is then amplified by PCR technique [5].
3. *Asymmetric PCR*: In this technique unequal concentration of primers is used. The great excess of primers is used for the targeted DNA strand that we need to amplify. As the reaction proceeds, only the adequate amount of primer in the reaction mixture produces the particular DNA strand in excess. Therefore ultimately single-stranded DNA (ssDNA) is formed as PCR product. As the reaction is slow and goes on arithmetically, so many more cycles are needed in this technique. Asymmetric PCR is used for DNA sequencing and hybridization as only one strand is needed in such conditions [6].

Disadvantages or limitations:

- The ssDNA is vulnerable to damage by many physical and chemical factors, and a more stable second structure may form.
- Different ssDNA may be formed even in the same reaction.
- It needs more thermal cycle.

4. *Hot start PCR* [7]: Normally DNA polymerase acts in the room temperature and even in the ice pack. Thereby there always remains the possibility of spurious products. In hot start PCR technique, the DNA polymerase is unreactive at the lower temperature and works only at higher temperature. This is done by conjugating an inhibitor with the polymerase enzyme, and in the higher temperature, the inhibitor is free from the polymerase enzyme and allows it to work.

   The various ways to do hot start PCR:

   - *Withheld the key agents until the end of initial denaturation process.*
   – DNA polymerase enzyme or magnesium cofactor
   - *Mechanical barriers of the reagents*:
   – DNA polymerase is encapsulated and is only released at higher temperature.
   – Wax barrier is used to separate the key components till the temperature is high.
   – Microfluidic devices are used to create barrier.
   - *Modification of DNA polymerase*:

- Antibodies are used to inhibit DNA polymerase activity at lower temperature, and it releases the enzyme in higher temperature.
- DNA polymerase enzyme is chemically modified so that it works only in higher temperature.
- The ligand is used that binds with DNA polymerase in a temperature-dependent way.
- Amino acid mutation is done in DNA polymerase enzyme to have reduced activity in lower temperature.

• *Accessory proteins*: The accessory proteins can be used that sequester primers at lower temperature.

Main advantage of hot start PCR: It reduces any unwanted PCR product.

5. *In situ PCR*: In case of in situ PCR, the reaction takes place within a cell on the glass slide. The PCR product is accumulated within the cell, so it is possible to locate the origin of the amplified DNA. The specially designed PCR machine is used to put the slide within it.

This technique is used to amplify the nucleic acid in the fixed tissue and cell instead in solution [8].

Use:

- Detection and location of virus within the tissue
- Detection and also localization of the cancer cells
- Demonstration of the genetic mutation in case of inherited genetic disease
- Demonstration of location of gene expression within the tissue

Advantages:

- High specificity
- High turnaround time
- Low background stain
- Avoidance of any radioactive elements

6. *Inverse PCR*: Inverse PCR (IPCR) amplifies anonymous DNA sequence. It helps to identify the flanking DNA sequence of the genome outside the boundary of the known target sequence [9].

There are four steps of IPCR (Fig. 20.2):

• *DNA isolation*: This is the initial basic step where genomic DNA is isolated from the sample and then cut into pieces by restriction endonuclease enzymes. The DNA is cut in such a way that the known sequences of DNA are in the inner region with unknown sequence in two sides.

• *Circularization of double-stranded DNA*: In this step the linear DNA molecule is circularized under low DNA concentration.

• *Reopening of the circular DNA*: With the help of endonuclease enzyme, the circular DNA is now cut to make linear DNA fragments. The known DNA sequence remains in the two ends of the unknown sequence.

• *Amplification of reverse DNA fragment*: Now with the help of the known primer, the known DNA sequence is amplified along with the attached unknown DNA sequence.

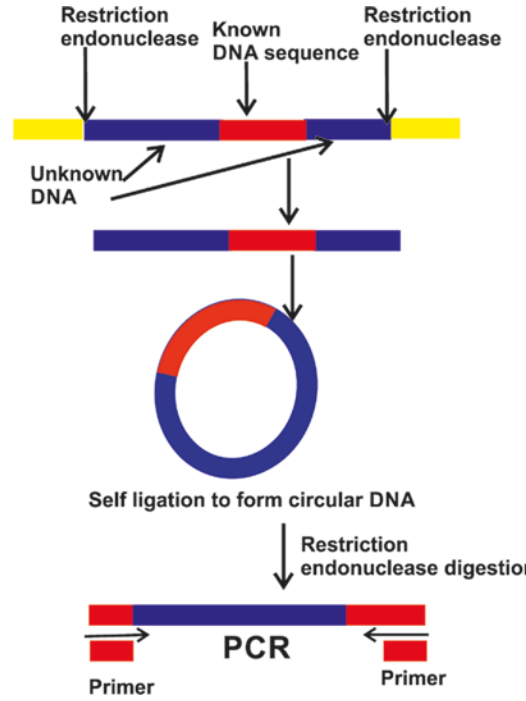

Fig. 20.2 Schematic diagram explains the basic principle of inverse PCR. At first DNA is isolated followed by circularization of double-stranded DNA. With the help of endonuclease enzyme, the circular DNA is cut to make linear DNA fragments with two fragments of known DNA in two ends. With the help of the known primer, the known DNA sequence is amplified along with the attached unknown DNA sequence

Applications of IPCR

- Identification of flanking sequence
- Identification of viral gene insertion within the genome
- Chromosomal rearrangement of oncogene

7. *Single-strand conformation polymorphism (SSCP)*:
   The basic principle of SSCP is that the single-stranded DNA has a specific conformation [10, 11]. Any alteration of the single base change due to mutation may lead to different migration pattern of the single-stranded DNA, and therefore in electrophoresis one can distinguish wild-type DNA from mutant DNA. The following steps are done in SSCP (Fig. 20.3):
   - PCR amplification of the target DNA.
   - The double-stranded DNA product is denatured.
   - The sample is cooled so that denatured single-stranded DNA undergoes self-annealing.
   - Electrophoresis is done to see the mobility of the single-stranded DNAs.

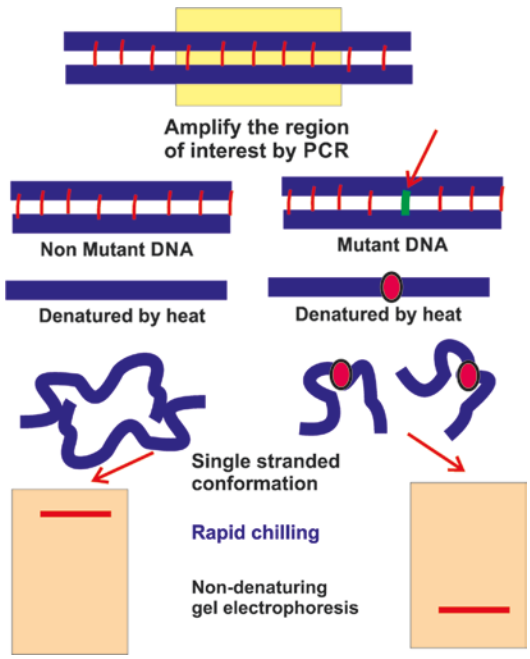

Fig. 20.3 Schematic diagram explains the basic principle of single-stranded conformation polymorphism. Alteration of the single base change due to mutation leads to different migration pattern of the single-stranded DNA in electrophoresis

   Application: The detection of single base change mutation and polymorphism in essential hypertension, carcinoma, diabetes, etc.

8. *Real-time PCR*: Real-time PCR is also known as quantitative PCR (qPCR) as it constantly monitors the quantity of the amplified DNA in the reaction process [12, 13]. In case of qPCR, the amplified DNA is fluorescently labelled, and the emitted fluorescent is directly proportional to the amount of the amplified fluorescent dye. Therefore in each cycle, the amount of the product can be directly monitored, and it is also possible to quantitate the initial amount of the target DNA in the sample.
   Mechanisms to quantitate the amplified DNA:
   - *Hydrolysis of the probe* (Fig. 20.4): In this real-time TaqMan® assay technique, we

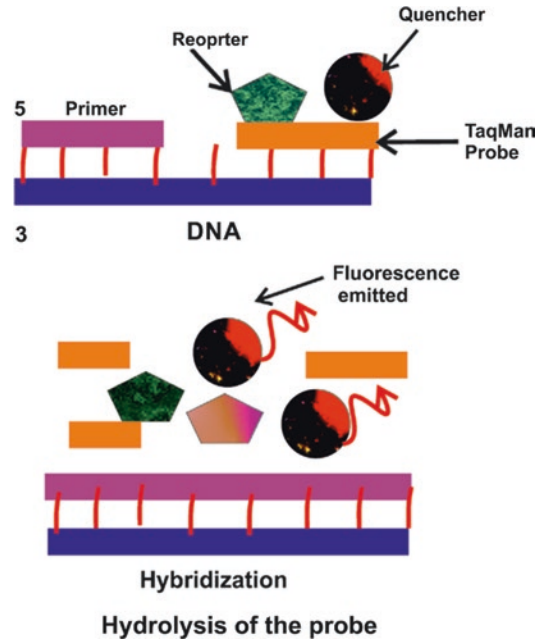

Fig. 20.4 Schematic diagram shows the basic principle of TaqMan probe using in PCR. The "TaqMan" probe is attached with a fluorescence reporter dye and a quencher dye. In case of intact TaqMan probe, the reporter dye and the quencher dye remains in close proximity, and no fluorescence is emitted. During PCR the endonuclease breaks down the "TaqMan" probe, and the reporter dye is away from quencher dye that allows emission of fluorescence which is directly proportional to the amount of PCR products

use a "TaqMan" probe. This is an oligonucleotide probe which is attached with a fluorescence reporter dye at its 5′ terminal and a quencher dye at the 3′ terminal end. In case of the proper target sequence, this probe anneals with one of the target sequence of DNA template. The "TaqMan" probe is cleaved by Taq polymerase during PCR. When the "TaqMan" probe is intact, the reporter dye and the quencher dye remain in close proximity, and therefore fluorescence emitted from the reporter dye is absorbed by the closely placed quencher dye. So no fluorescence emitted. During PCR the endonuclease breaks down the "TaqMan" probe, and the reporter dye is away from quencher dye that allows emission of fluorescence. The increased intensity of fluorescence is directly proportional to the amount of PCR products.

- *DNA-binding dye* (Fig. 20.5): In this technique DNA-intercalating agents SYBR® Green are used. The SYBR® Green dye molecules do not exhibit any fluorescence in solution. However, the dye molecules emit fluorescence when they are intercalated within the double-stranded DNA that is formed after the primer extension and polymerization. After each cycle the emitted fluorescence from the polymerized DNA is measured to estimate the total amount of the amplified DNA.

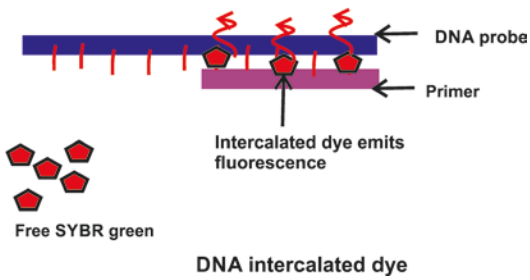

Fig. 20.5 Schematic diagram shows the basic principle of using DNA-binding dye to quantitate the PCR products. The free SYBR Green dye molecules do not exhibit any fluorescence in solution. In PCR extension, the dye molecules are intercalated within the double-stranded DNA and emit fluorescence

- *Dual hybridization* (Fig. 20.6): In this technique two hybridization probes are applied. The first probe is attached with a donor fluorophore at the 3′ end, and the other probe carries an acceptor fluorophore at the 5′ terminal. In denaturation step there is no emission of fluorescence as any fluorescent emission by donor fluorophore is degraded by the acceptor fluorophore. In the annealing stage, the donor and the acceptor fluorophore probes hybridize to the target DNA sequence, and they are adjusted in head to tail position so that donor fluorophore comes in close contact with the acceptor fluorophore. This allows fluorescence resonance energy transfer. The intensity of the fluorescence is measured which is directly proportional to the amount of PCR products.
- *Molecular beacons* (Fig. 20.7): In this case the hybridized probe is designed like

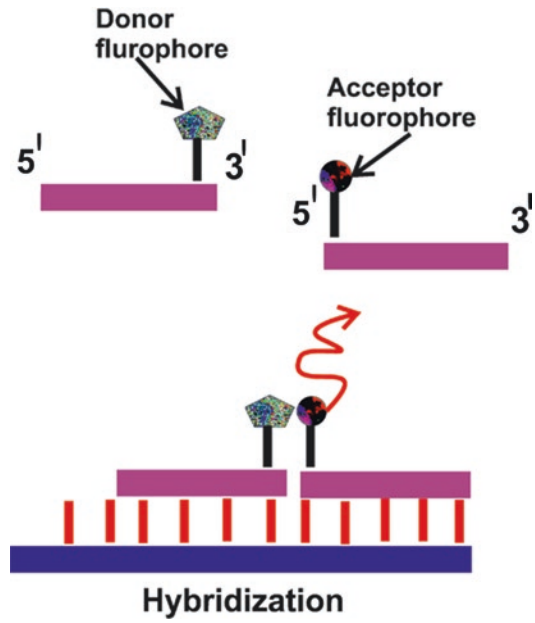

Fig. 20.6 Schematic diagram explains the basic principle of dual hybridization technique to quantitate the PCR products. In this technique the first probe is attached with a donor fluorophore, and the other probe is attached with an acceptor fluorophore. During PCR process the donor and the acceptor fluorophore probes hybridize to the target DNA sequence and come in close contact. This allows fluorescence resonance energy transfer

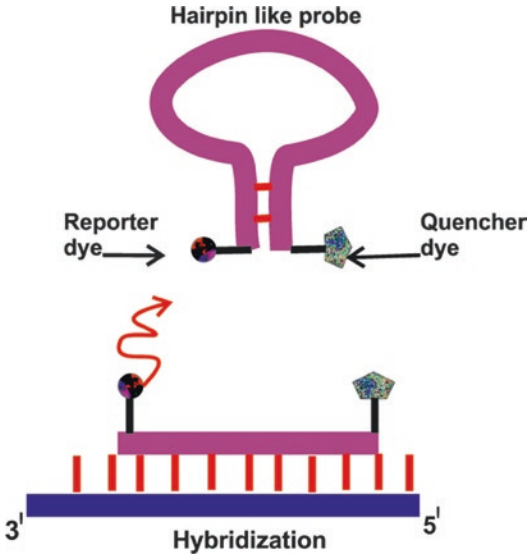

Fig. 20.7 Schematic diagram shows the basic principle of molecular beacon technique to quantitate the PCR products. Here the hybridized probe is designed like a hairpin-like loop, and the reporter and quenching dyes are attached in the two ends of the loop. The close proximity of them prevents the emission of any fluorescence. During annealing of the hybridized probe, the hairpin loop becomes a straight probe, and the reporter and quenching dyes stay away that allows emission of fluorescence

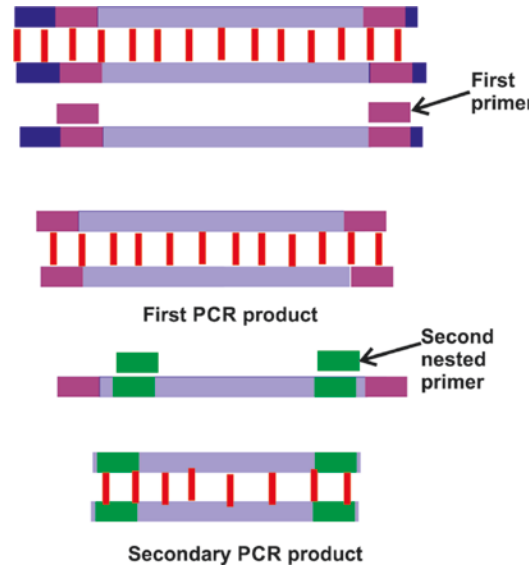

Fig. 20.8 Schematic diagram shows the basic principle of nested PCR. Here more than two pairs of primers are used for DNA amplification. At first primer is used for the conventional PCR of the sample. In secondary PCR the product of the first PCR is used as the target of the second set of primers

a hairpin-like loop. The reporter and quenching dyes are attached in the two ends of the loop, and the close proximity of them prevents the emission of fluorescence. At the time of annealing of the hybridized probe, the hairpin loop becomes a straight probe, and the reporter and quenching dyes stay away. This allows emission of fluorescence. The increased fluorescent intensity is measured to quantify the PCR products.

9. *Nested PCR* (Fig. 20.8): In case of nested PCR, more than two pairs of primers are used for DNA amplification. The first PCR is a conventional PCR, and the primer is used for the DNA template of the sample. In secondary PCR the product of the first PCR is used as the target of the second set of primers. The DNA sequence of secondary PCR is different, and therefore there is no chance of undesired PCR product formation.

20.5 Applications of PCR

The various applications of PCR in clinical area are described below (Table 20.2) [14, 15]:

(a) *DNA sequencing*: PCR is often used in case of DNA sequencing.
(b) *Diagnosis of infection*: PCR is widely used for the diagnosis of viral, bacterial and parasitic infection. As mentioned before Q-PCR can quantitate the viral load in the body [16]. PCR rapidly detects the tuberculosis within a few hours, whereas the culture of mycobacteria takes few weeks to develop. As a sensitive technique PCR is able to detect tuberculosis in the early latent phase.
(c) *Diagnosis and prognostic information on cancer*: PCR technique is extensively used in the field of cancer:
   - *Mutation detection*: PCR is applied to detect mutation of the oncogenes and

Table 20.2 Applications of PCR in basic research and clinical field

Applications of PCR	
Basic research	Clinical applications
• DNA sequencing	• Diagnosis of infections
• Bioinformatics • Classification of organisms • Gene expression studies	• Cancer – Detection of chromosomal abnormalities – Genetic mutation – Detection of minimal residual disease
• Drug discovery	• Genetic disease: intranatal detection of inherited genetic disease, e.g. Down's syndrome, Gaucher's disease, etc.
	• Forensic pathology: – Paternity detection – Identification of mutilated body – Crime site investigation
	• Gene therapy

tumor suppressor gene such as mutation in p53, c-myc, ras gene, etc. [17, 18].

- *Chromosomal changes*: PCR helps to identify the specific chromosomal changes such as chromosomal translocation, gene rearrangement, loss of heterozygosity, etc. [16].
- *Monoclonality detection*: PCR can detect B and T cell gene rearrangement and thereby can prove the monoclonality in doubtful case of lymphoma [17].
- *Minimal residual disease*: In case of follow-up of a cancer case, PCR particularly Q-PCR can detect and quantitate certain genetic change to detect any minimal residual disease of a patient [18].

(d) *Genetic diseases*: PCR technique is very helpful to detect various genetic diseases such as Down's syndrome, cystic fibrosis, Gaucherec disease, etc. The main advantage of PCR technique is that it can bypass the aggressive placental bed biopsy to detect these inherited diseases. The minute amount of foetal cells collected from the mother's blood or cervical mucosa are enough to reach at a diagnosis [19].

(e) *Forensic pathology*: PCR technique is helpful in forensic pathology in different ways:
- To detect paternity of the child
- To identity of the corpse or mutilated body
- To identify the criminal from the crime site and biological materials of the criminal.

(f) *Gene therapy*: PCR helps to engineer the specific gene to introduce in the diseased person to cure various diseases [20].

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21 Fluorescent In Situ Hybridization Techniques in Pathology: Principle, Technique and Applications

21.1 Introduction

Fluorescent in situ hybridization technique (FISH) is also known as interphase cytogenetics. In this technique the double-stranded DNA is at first converted into single-stranded DNA, and then subsequently a fluorescent-tagged probe is used to visualize the target DNA part [1–3].

Advantages FISH techniques have many advantages over the conventional cytogenetic techniques (Box 21.1). It is possible to do FISH on the paraffin-embedded tissue material, and therefore it can be used in archival materials. FISH technique bypasses the tedious cell culture technique. Most importantly cytogenetic abnormality of the cells can be demonstrated along with the morphology of the cell, and these two can be correlated.

Box 21.1 Advantages of FISH

- No need of cell culture.
- FISH can be done on paraffin cell block material.
- Archival tissue can be used for FISH.
- Morphology of the cell can be seen along with cytogenetic abnormalities.
- High resolution.
- The slide can be stored for long time.
- Fluorescent tags are safe and simple.

Limitations of FISH: FISH has following limitations:

- The FISH technique can be used only in the case of known chromosomal abnormalities as we use only the specific probes.
- It is not suitable for a screening test as only known chromosomal probes are used, whereas in cell culture or conventional technique, we may get a wide range of chromosomal abnormalities.
- FISH does not give any allele-specific information.

Applications of FISH:

- *Gain and loss of chromosome*: FISH is helpful to detect total gain or loss of chromosome such as trisomy 12 in chronic lymphocytic leukaemia which can be detected by FISH in cytology or histology sample.
- *Chromosomal rearrangements*: FISH is helpful to identify typical chromosomal translocation such as t (11; 22) (q24; q12) in Ewing's/primitive neuroectodermal tumor [4].
- *Gene amplification*: Gene amplification such as HER-2 in breast carcinoma can be detected by FISH.
- *Gene deletion*: Gene deletion such as 9p21 deletion in urothelial cell carcinoma can be detected by FISH.

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_21

- *Disease monitoring*: To assess the progression or regression of disease and also to identify the minimal residual disease.

21.1.1 The Principles of FISH

The basic principles of FISH are (Fig. 21.1):

- To convert double-stranded DNA into single-stranded DNA.
- DNA probes tagged with fluorescent dyes are also made to single stranded.
- The florescent-tagged single-stranded DNA probes are allowed to bind with the corresponding single-stranded DNA.
- The hybridized probe-target DNA complexes are visualized by fluorescence microscope**.**

Probes Applied in FISH Three types of probes are applied for FISH and they include:

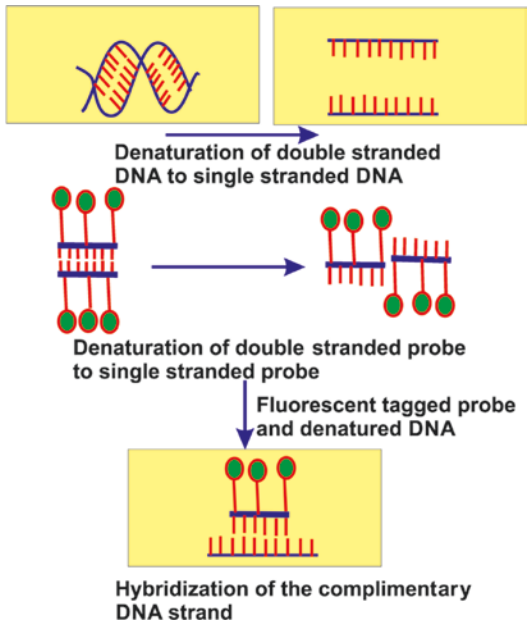

Fig. 21.1 Schematic diagram shows the basic principle of FISH technique. At first double-stranded DNA and also primer DNA are converted into single-stranded DNA. DNA probes are tagged with fluorescent dyes and are allowed to bind with the corresponding single-stranded DNA

- *Centromeric probes or chromosome enumeration probes (CEP)*: CEP is directed against the alpha or beta satellite repeat sequence of the centromeric region of the chromosome. Therefore it helps to enumerate the number of chromosome. The CEP can detect the monosomy or aneuploidy.
- *Locus-specific identifier (LSI) probes*: LSI probe is used to demonstrate the other specific region of the chromosome. These probes are used for the demonstration of various structural abnormalities such as gene deletion, translocation, etc. LSI also helps in the detection and localization of gene and the demonstration of gene amplification.
- *Whole chromosome probes (WSP)*: The whole chromosome probes consist of multiple smaller probes with complimentary DNA sequence of different parts of a single chromosome. Multiple WSP can be used to stain a whole chromosome. Therefore using different fluorochrome for different chromosome, one can paint each chromosome with different colour. WSPs are used to demonstrate chromosomal structural abnormalities (translocation or deletion).

21.2 Steps to Do FISH [5–7]

Histology and Cytology Specimen:

1. *Fixation*: The cytology slides are fixed with 4% paraformaldehyde for 10 min. Histology tissues are already fixed in 10% buffered formalin.
2. *Deparaffinization of the histology section*:
   (a) Cut 5-μ-thick section.
   (b) Deparaffinize the histology section by baking the slide overnight in 56 °C.
   (c) Keep the slide in xylene for 10 min: two changes.
   (d) Dehydrate by treating in 70% and 100% ethyl alcohol twice for 5 min.
   (e) Dry the smear in hot plate.
3. *Pretreatment*: 20 μg/ml proteinase K for 5–15 min

- Dehydrate the smear by dipping in 70%, 80% and 95% ethyl alcohol and dry the smear.
- Treat the smear with proteinase K solution (20 µg/ml) for 15 min in room temperature.

- (Proteinase K solution preparation: add 32 µl of proteinase K solution (25 mg/ml proteinase K solution) in 40 ml 2× SSC, pH 7.4.)
- Gently wash the slide in deionized water.
- Dehydrate the smear.

Saline sodium citrate (SSC): Add

Sodium chloride	175.3 g
Sodium citrate	88.2 g
Distilled water	800 ml

Keep pH 7.2 by adding drops of 10 N solution of NaOH. Now add water and make it 1 litre.

4. *Denaturation*:

   Denaturing of target DNA: Denature DNA of the target cells in the smear by treating the smear with denaturing solution for 2 min time at 72 °C.

   Denaturing solution: 70% formamide in 2× SSC (add 10 ml of double-distilled water, 5 ml of 20× SSC, 25 µl of 250 mM EDTA and 35 ml of formamide).

   Denaturing of probe DNA: Add 1 µl of labelled probe with 9 µl of 65% formamide solution, 10% dextran sulphate in 2× SSC.

   Now heat the mixture at 75 °C for 5–6 min.
5. *Hybridization*:
   - Add 10 µl of denatured probe solution over the slide.
   - Put a coverslip over the smear and close the margins of the coverslip.
   - Incubate it at 37 °C for 1 day (24 h).
6. *Post-hybridization*:
   - After the incubation, remove the coverslip gently, and rinse the slides in SSC for 5 min twice.
   - Put the slides in a Coplin jar filled with pre-warmed SSC at 70 °C.
   - Keep the slide in Coplin jar filled with SSC at room temperature.
7. *Visualization*: If the probes are directly labelled with the fluorochrome dye, then no further procedure is needed. In that case, counterstain the slide by 5 µl DAPI/antifade solution. Now visualize the cells by an epifluorescence fluorescence microscope.

21.3 Troubleshooting

Table 21.1 has described the problems in FISH techniques and their remedies.

21.3.1 Different Types of FISH

1. *Three-dimensional FISH (3D FISH)*: In this type of FISH, multiple images of the nuclei are taken, and with the help of a suitable software, a three-dimensional image is made [8]. 3D FISH helps to study the topology of the genes in respect to the chromosomal territory within the nucleus.
2. *Living cell cytogenetics (four-dimensional FISH)*: Fluorescent-tagged nucleotide can be incorporated into DNA that may help in the simultaneous visualization of DNA distribution and genomic organization in the living cells [9].
3. *Multi-coloured FISH (M-FISH)/spectral karyotyping (SKY)*: In the case of SKY technique, DNA is tagged with different fluorochrome dyes (the chromosome-specific painting probes), and all the chromosomes are stained. The different chromosome therefore takes different colour (Fig. 21.2). There are three essential steps of M-FISH:
   (a) *Hybridization*: The fluorescent-tagged whole chromosome probes (WCP) are hybridized with the metaphase chromosome spread. WCPs consist of multiple probes tagged with spectrally different fluorochrome in combinatorial manner.
   (b) *Visualization and image acquisition*: In this step the images of the chromosomes are visualized by the fluorescence microscope with attached filters. Subsequently the images are acquired by the digital camera and appropriate software.

Table 21.1 Troubleshooting of FISH technique

Problems	Possible cause	Remedies
Weak signal or no signal	• Poorly digested tissue section	• Check the pH of the proteinase K solution • Check the temperature of the tissue digestion step • Make fresh proteinase K solution
	• Low concentration of the probe	• Maintain the optimum concentration of the probe
	• Faulty hybridization step	• Check the total time of hybridization (at least 14 h) • Check the incubation temperature
	• Loss of DNA	• The solution should be DNase-free
	• Faulty microscope	• Check the bulb of the microscope
Intense background staining	• Non-specific attachment of the probe and interspersed repetitive sequences of DNA	• Add species-specific excess amount of Cot-1 DNA • Wash the slide stringently after the hybridization step
Variable staining in different areas	• Uneven distribution of the hybridization solution	• Avoid any air bubbles under the coverslip
Tissue morphology is poorly preserved	• Overdigestion of tissue	• Check the digestion time and reduce the time period
	• Improper fixation	• New sample has to be taken
Speckled appearance	• Less stringent solutions	• Maintain stringent concentration of the buffer solution, temperature, etc.

(c) *Analysis*: Finally the images are analysed with the help of the specialized software to find out any structural chromosomal abnormalities.

Steps of M-FISH [10, 11]

Pretreatment of the metaphase spread by trypsin:

- Incubate the slide in pepsin at 37 °C for 5 min.
- (Pepsin 1: 20,000 in 10 mM HCl.)
- Wash the slide in PBS: 5 min.
- Fix the slide by 1% formaldehyde.
- Wash again in PBS: 5 min.
- Dehydrate in serially graded alcohol (70%, 90% and 100%) for 2 min each.

Denaturation of chromosome

- Incubate the slide in 50 ml denaturing solution within a Coplin jar for 3 min at 72 °C.
- Immediately dip the slide in ice-cold ethanol 70%, 90% and 100% each for 2 min.
- Dry the slide.

Denaturation of probe

- Centrifuge the M-FISH probes.
- Mix the contents gently and take 10 µl probe solution for each slide in an Eppendorf Tube.
- Incubate the Eppendorf Tube at 80 °C for 5–7 min to denature the probe.

Hybridization

- Add 10 µl denatured M-FISH probe solution over the denatured chromosome preparation.
- Put coverslip over the smear and seal the margins of the coverslip with rubber cement.
- Keep the slide in a humidified chamber for 2 days at 37 °C.
- Remove the slide from the chamber, and take out the coverslip by removing the rubber cement.
- Wash the slides in SSC at 72 °C for 2 min.
- Counterstain with DAPI and place a coverslip.
- The slide is now ready to visualize in the fluorescence microscope.

4. *Comparative genomic hybridization (CGH)*: CGH provides the global view of gain or loss of chromosome of the tumor genome [12].

Basic principles (Fig. 21.3):

Tumor DNA is extracted from the sample and labelled by green fluorochrome dye.

Normal DNA from the control is also extracted and labelled by red fluorochrome dye.

The mixture of both green-labelled tumor DNA and red-labelled control normal DNA is mixed and allowed to hybridize on the normal metaphase chromosome.

With the help of digital image analyser, the green/red ratio is measured.

Excess green fluorescence represents chromosomal gain or amplification.

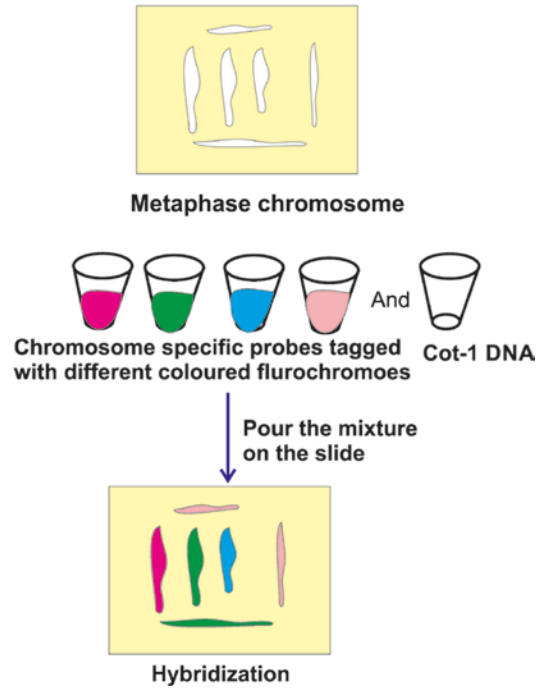

Fig. 21.2 Schematic diagram shows the basic principle of spectral karyotyping. DNA is tagged with different fluorochrome dyes, and the different chromosome takes different colour

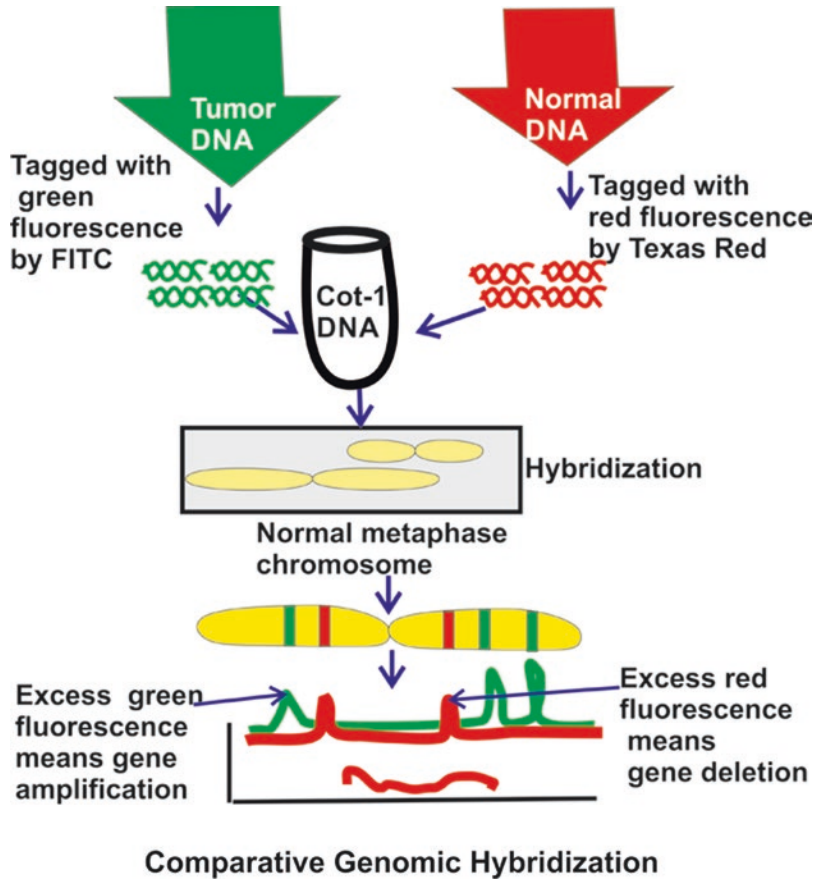

Fig. 21.3 Schematic diagram shows the basic principle of comparative genomic hybridization. Tumor DNA is labelled by green fluorochrome dye, and normal DNA from the control is labelled by red fluorochrome dye. They are allowed to hybridize on the normal metaphase chromosome. With the help of digital image analyser, the green/red ratio is measured. The excess green fluorescence represents chromosomal gain or amplification, whereas excess red fluorescence represents chromosomal loss

Excess red fluorescence represents chromosomal loss.

Advantages of CGH: CGH technique is helpful in tiny amount of micro-dissected tissue. The technique can be done without any prior knowledge of the chromosomal abnormalities in the tumor DNA.

Li mitations of CGH: CGH is not helpful if there are chromosomal abnormalities without any gain or loss of genetic material. Similarly from CGH we do not get any information on the structural changes of the chromosome. CGH is much less sensitive than PCR, and the result may be changed due to contamination of normal cells with tumor cells.

Box 21.2 Advantages and limitations of CGH

Advantages

- Tiny micro-dissected tissue can be processed for CGH.
- No need of details of chromosomal abnormalities of tumor tissue.

Limitations

- Ineffective technique if there are chromosomal abnormalities without any gain or loss of genetic material
- No information of any structural abnormalities
- Tedious and prolonged process

21.3.2 CGH Method [13]

Preparation of probe mixture:

1. Add the following substances in microcentrifuge tube.
   - Fluorescein-tagged test DNA (10 µl 20 µg/ml)
   - Texas red-labelled reference probe DNA (10 µl 20 µg/ml)
   - Human Cot-1 DNA (20 µl 1 µg/ml)
2. Add 1/10th volume of 3 M sodium acetate (pH 5.2) and mix them.
3. Add 2.5 volume of 100% ethyl alcohol (ice cold) and vortex again.
4. Remove the supernatant fluid.
5. Resuspend the pellet by adding 10 µl hybridization mixture.

Denature target metaphase smear and hybridization mixture

6. Fix the metaphase slide by 4% paraformaldehyde for 15 min at 4 °C.
7. Rinse the slide in PBS.
8. Incubate the slide in proteinase K solution for 5 min at 37 °C.
9. Rinse in PBS for 5 min.
10. Denature the metaphase spread smear: Keep the slide in preheated Coplin jar in water bath containing denaturing solution at 75 °C for 5 min.
11. Dehydrate the slide by graded alcohol and dry in air.
12. Simultaneously denature the DNA samples: Heat the sample at 75 °C for 5 min. Immediately incubate the sample at 37 °C for 20 min.

Hybridization

13. Pour 10 µl of the probe mixture on the metaphase smear. Cover the area by a coverslip and seal the edges by rubber cement.
14. Incubate the slide in a humid chamber at 37 °C for 48 h.

Washing

15. Peel off the rubber cement.
16. Wash the slide twice in SSC for 5 min each.
17. Wash the slide twice by pre-warmed hybridization buffer at 45 °C for 10 min.
18. Wash the slide in PBS for 5 min at 37 °C.
19. Dehydrate the slide by graded alcohol and air-dry the smear.

Counterstain

20. Pour 8 µl DAPI and apply coverslip over the smear.

Analysis

21. Analyse the slide under fluorescence microscope with attached digital camera and appropriate software.

21.3.3 Array-Based CGH [14]

The basic principle of array-based CGH (aCGH) is similar to CGH. However aCGH uses specific target DNA sequence instead of metaphase chromosome. Microarray plate with multiple wells contains genomic bacterial artificial chromosome or cDNA in the array.

Basic steps of aCGH (Fig. 21.4)

1. Tumor DNA is extracted and labelled with a green fluorochrome dye.
2. Control DNA from healthy person is extracted and labelled with red fluorochrome dye.
3. The mixture of the above two samples is hybridized with multiple specific DNA probe in the microarray plate.
4. The hybridization reaction plate is read by image analyser with appropriate software.

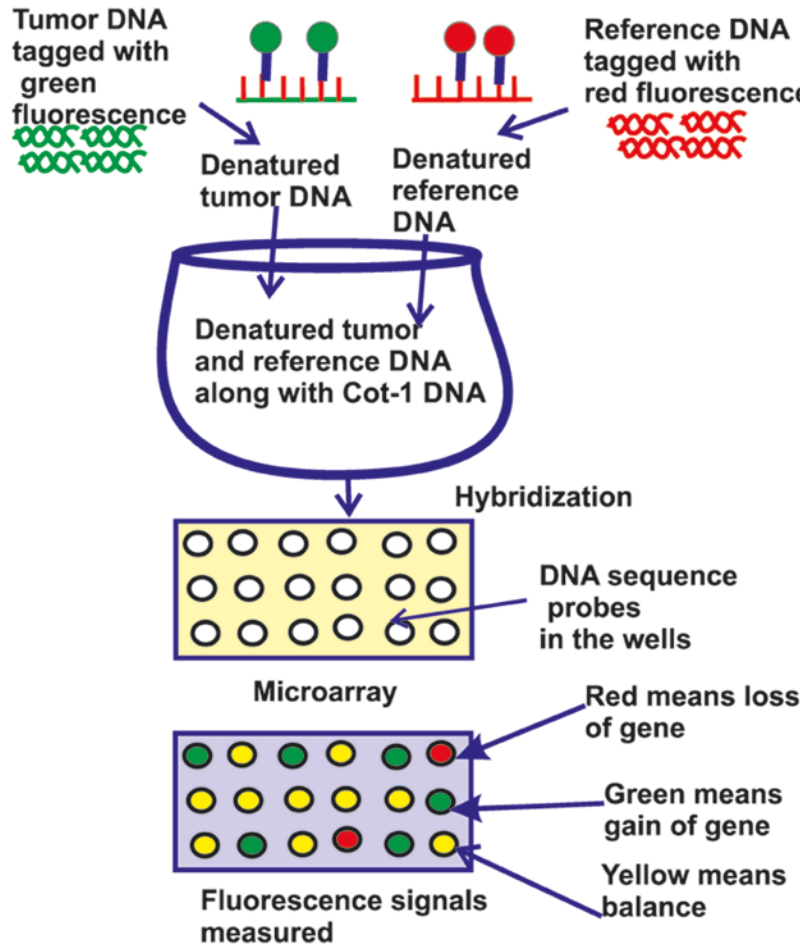

Fig. 21.4 Schematic diagram shows the basic principle of array-based comparative genomic hybridization. Tumor DNA is labelled with a green fluorochrome dye, and the control DNA is labelled with red fluorochrome dye. The mixture of them is hybridized with multiple specific DNA probe in the microarray plate, and the hybridization reaction plate is analysed

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22 Tissue Microarray in Pathology: Principal, Technique and Applications

22.1 Introduction

Tissue microarray (TMA) is a novel technology that helps to study a large number of tissue samples in a single section [1–3]. In this technique few hundreds to thousands of tissue samples from the different paraffin block can be taken and are precisely put into a recipient paraffin block. TMA helps to perform immunocytochemistry (ICC), fluorescent in situ hybridization (FISH) or RNA analysis in the single section from the recipient block. Therefore the technique is faster and cheaper than the conventional method.

22.2 Tissue Microarray Technique

At first, haematoxylin- and eosin-stained section from the donor block is studied, and the representative area of the donor block is marked. With the help of tissue microarray instrument, core tissue biopsy (0.6–2 mm diameter) is taken from the donor paraffin block and is placed in an empty paraffin block (the recipient block) in precise manner (Fig. 22.1). The recipient block can accommodate few hundreds to thousand specimens. The co-ordinates of the core biopsies in the recipient block are recorded typically in a spreadsheet (preferably in Microsoft Excel file). Now with the help of a microtome, 5 μ sections are cut from the TMA block to produce TMA slide (Fig. 22.2). The section now can be used for IHC, FISH or other molecular studies.

22.3 TMA Construction and Generation of Grid

The construction of the TMA and the generation of the grid depend mainly on the type of the study.

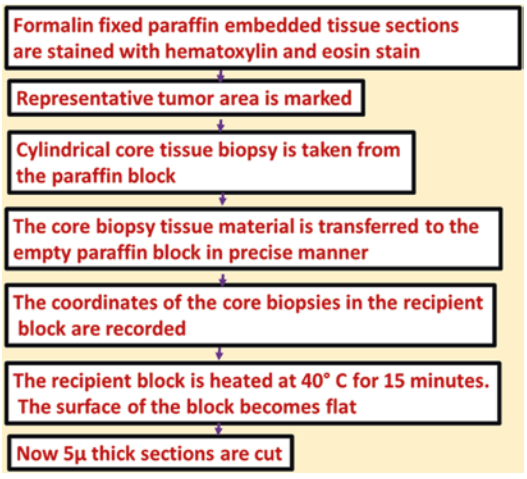

Fig. 22.1 Basic steps of tissue microarray are described in this diagram

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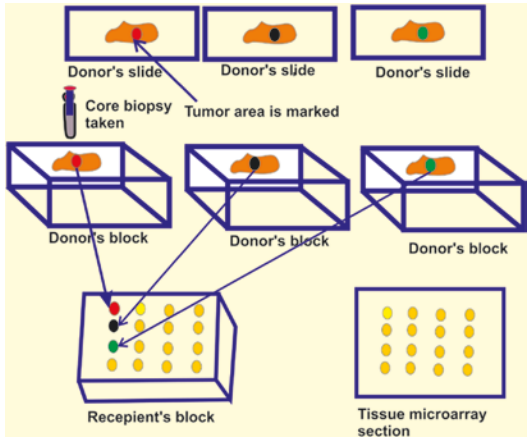

Fig. 22.2 The schematic diagram shows the detailed steps of tissue microarray. With the help of tissue microarray instrument, core tissue biopsy is taken from the donor paraffin block and is placed in an empty paraffin block (the recipient block)

- At first make a list of suitable cases.
- Study all the section of these cases.
- Review the cases and reclassify if needed. Then mark the representative area on the stained section on glass slide. It is preferable to use different colour for different diagnostic area such as green for normal area, red for tumor and black for in situ carcinoma.
- Collect the representative block of the slides and identify the area.

22.4 Designing the Grid [4]

- Capital letters indicate the quadrant, and small letters indicate the co-ordinate of the tissues in the recipient's block (Fig. 22.3).
- The primary data are entered for each tissue core such as biopsy number, location, diagnosis, grade of tumor, stage, etc. The data is usually entered in a Microsoft Excel file (Fig. 22.3).
- *Protection wall*: The peripheral boundary walls of the TMA are made of single row of core tissue (Fig. 22.4). These core tissues may consist of any tissue and they are not analysed.
- *Control tissue*: Positive and negative control tissues are placed in asymmetric location (see green dots in Fig. 22.4).
- *Tumor tissue*: The tumor tissues are placed in small groups (see the red dots in Fig. 22.4).
- *Normal heathy tissue*: In between the groups of tumor tissue, the matched normal healthy tissues are placed in one or two rows (see the black dots in Fig. 22.4). The presence of these normal tissue rows may help in the better orientation of the tumor tissues.
- *Empty cores in small row*: Intentionally few cores in a row should be kept empty to immediately identify the orientation of TME grossly.
- The different disease processes can be grouped together such as carcinoma cases; in situ carcinoma and normal cases are clubbed in different groups.

Diameter of the Core The diameter of the punches of the core biopsy may vary from 0.6 to 2 mm. In fact most of the people take 0.6 mm diameter core that includes 0.14 square mm tissue.

Number of Cores in TMA In a 2.5 × 4.5 cm block, at least 1000 cores can be adjusted. However it is preferable not to take more than 500 cores in a single block.

Recipient Block The recipient block is made of fresh molten paraffin. The average size of the block is 2.5 × 4.5 cm. Adequate precautions should be taken to avoid any bubbles within the block.

Automated TMA Presently automated tissue microarray machine is available that bypasses the tedious manual punching procedure. It helps to design the array design or layout, and the machine automatically creates holes in the paraffin wax and places the donor tissue in the hole of the recipient paraffin wax.

5.Advantages of TMA There are several advantages of TMA that include (Box 22.1):

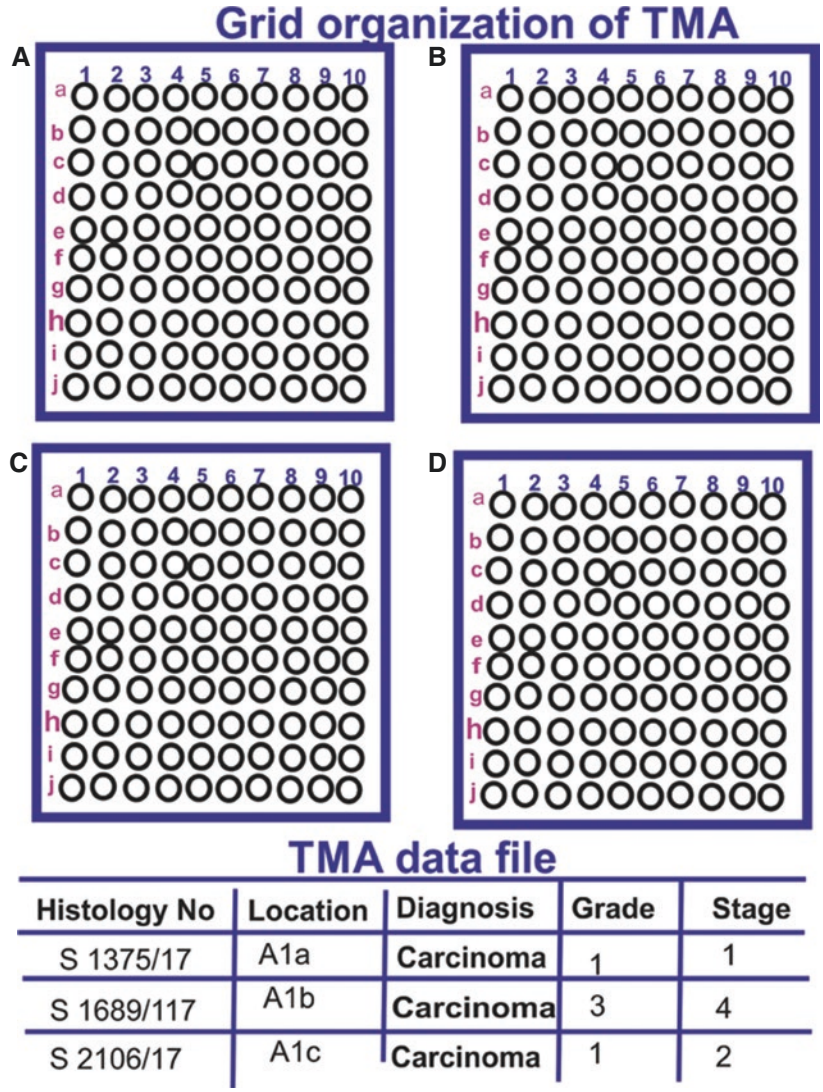

Fig. 22.3 The schematic diagram of the organization of the grid for tissue microarray. The capital letters indicate the quadrant, and small letters indicate the co-ordinate of the tissues in the recipient's block. The primary data for each tissue core such as biopsy number, location, diagnosis, grade of tumor, stage, etc. are entered in a Microsoft Excel file

1. *Amplification of the resource*: Ordinarily from a standard 5 mm thick section of tissue, we can get maximum 100 sections for study. Whereas depending on the size of the tissue in the original block, at least 200 core biopsies can be taken to make TMA block. After the construction of the TMA, we can cut at least 1000 sections of 3 μ thickness from the 5 mm thick array block. Therefore we get a total 1000 × 200 means 200,000 sections from the limited resource.
2. *Uniformity in staining conditions*: At the time of conventional staining, the different tissue sections are stained at different time (such as 10 batches of 20 slides each), and the slide-to-slide variation may occur due to several variables such as antigen retrieval, concentration of different reagents, incubation period, washing time, etc. However in case of TMA, each tissue section consists of 100–1000 core biopsies from the different patients, and the single section is stained that avoids all the slide-to-slide variability.
3. *Faster, cheaper and reduction of manpower*: In TMA a single slide requires less reagents, labour and time. Therefore, TMA saves cost, time and total work force.

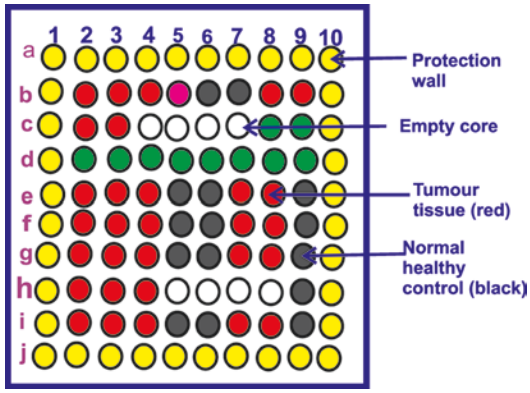

Fig. 22.4 The schematic diagram shows the elaborated design of the grid for tissue microarray. The peripheral boundary walls of the TMA are made of single row of core tissue. Positive and negative control tissues are placed in asymmetric location (green dots). The tumor tissues are placed in small groups (red dots). Normal healthy tissues are placed in one or two rows (black dots)

4. *Original block can be preserved*: Only a few core biopsies from the original block are sufficient to make TMA. The original block can be preserved and can be used for further sectioning.
5. *Effective in quality assurance program*: Due to significant amplification of the laboratory material, TMA can be used for external and internal quality control program. The TMA section can also be used for standardization of reagents for positive control.
6. The whole analysis of TMA is now automated and computer can keep track of the huge data.

Box 22.1: Advantages and Limitations of TMA

Advantages:

- Amplification of the resource: Nearly about 100,000 times amplification is possible.
- Uniformity in staining conditions: Overall uniformity of the staining is feasible.
- Faster and cheaper: When large number of core tissue is processed, it becomes cheaper.
- Preservation of the original block is possible: Even after multiple core biopsies, the donor tissue block is useable for the diagnosis.
- Effective in quality assurance program.

Limitations:

- Tissue heterogeneity
- Not suitable in small series with occasional use
- Loss of the tissue
- Confusion on orientation

22.5 Limitations of TMA

The main limitation of TMA is tissue heterogeneity.

1. *Tissue heterogeneity*: This is one of the main concerns of TMA. The tumor may vary from area to areas such as Hodgkin lymphoma which may have different morphologies in different areas. Therefore small 2 mm tissue may not represent the whole tumor and finding may vary. However, several studies have shown that TMA and whole tissue data are almost similar [5, 6].
2. *Less cost-effective in small series of cases*: TMA is not very effective if it is done once in a while in a small series of cases.
3. *Prone to loss the tissue*: The core biopsy tissues may be lost due to its tiny size. Tissue rich in keratin, bone or cartilages are likely to be lost.
4. *Disorientation of the core biopsy tissue*: Due to large number of core biopsies, there is a chance of disorientation when TMA is done manually. Rows of empty core tissues may help in the immediate orientation of the tissue.

22.6 Clinical Applications of TMA

TMA helps to implement various molecular discoveries in clinical areas. The novel genes can be identified by DNA microarray technique, and subsequently these gene products can be validated by TMA [7, 8].

There are three different categories of TMA which are described in this respect:

1. *Different types of tumor for the novel markers*: Large varieties of tumor can be studied for the novel markers in an ultrashort time.
2. *Tumor progression array*: One particular tumor type is studied for its progression such as in a single section, one can study normal prostatic tissue, prostatic hyperplasia, intraepithelial neoplasia and carcinoma [9].
3. *Prognostic array*: Novel markers related to the prognosis of the malignant tumor can be studied by TMA.

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23 Sanger Sequencing and Next-Generation Gene Sequencing: Basic Principles and Applications in Pathology

DNA sequencing means the determination of the order of sequence of the base pair in DNA.

23.1 Sanger Sequencing

Sanger sequencing is named under Sanger F. This is the widely used commercial DNA sequencing [1].

Basic Principle In Sanger sequencing technique, 2′,3′-dideoxynucleotides are used for DNA synthesis. In the absence of 3′-hydroxyl group in 2′,3′-dideoxynucleotides, DNA cannot be synthesized further as no phosphodiester bond can be formed with the next dNTP, and the chain terminates (Fig. 23.1). The four DDNTPs (dideoxynucleotides phosphates such as ddATP, ddTTP, ddCTP, and ddGTP) are labelled by different fluorochrome dyes so that they are recognized by laser beam. Each fluorescent-labelled terminated fragment of DNA is recorded and from this data DNA sequence is assessed.

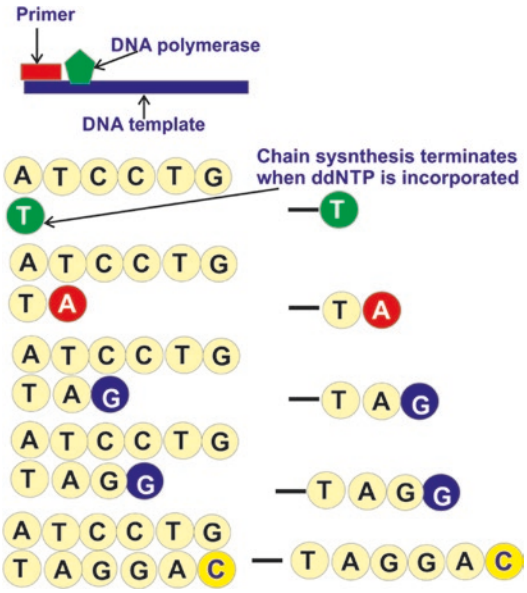

Fig. 23.1 Schematic diagram showing the principle of Sanger sequencing technique. Here 2′,3′-dideoxynucleotides are used for DNA synthesis, and in the absence of 3′-hydroxyl group, the DNA chain is terminated. The four DDNTPs are labelled by different fluorochrome dyes so that they are recognized by laser beam, and from this data DNA sequence is assessed

Reagents needed:

- Primers: Small piece of single-stranded DNA is used.
- DNA template: This chain is sequenced.
- DNA polymerase enzyme.
- dNTPs (dATP, dTTP, dCTP, dGTP).
- ddNTP (fluorescent labelled): All four ddNTPs such as ddATP, ddTTP, ddCTP and ddGTP are labelled with different fluorochrome dyes. These are chain-terminating nucleotides.

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Main steps:

- Mix the above reagents, and denature the complementary strands of DNA template by heating at 96 °C.
- Lower the temperature to 50 °C for annealing the primer with DNA template.
- Raise temperature to 60 °C for the elongation of DNA with the help of DNA polymerase. The DNA chain elongates till it incorporates fluorescent-labelled ddNTP. At that point the DNA chain elongation is terminated. The DNA chain is terminated with various lengths bearing the terminal-labelled ddNTP (fluorochrome-labelled A, T, C and G).
- The PCR cycle of DNA is repeated several times to have all the base position of the DNA template. At the end, variable lengths of DNA chains with terminal unique fluorescence labelled A, T, C and G are obtained.
- DNA chains undergone capillary gel electrophoresis, and then the coloured emission spectrum of all these fluorochrome-labelled DNAs is recorded by capillary gel electrophoresis (Fig. 23.2). With the help of a suitable software from the data of the terminal bases, the exact sequence of the bases in the DNA is generated.

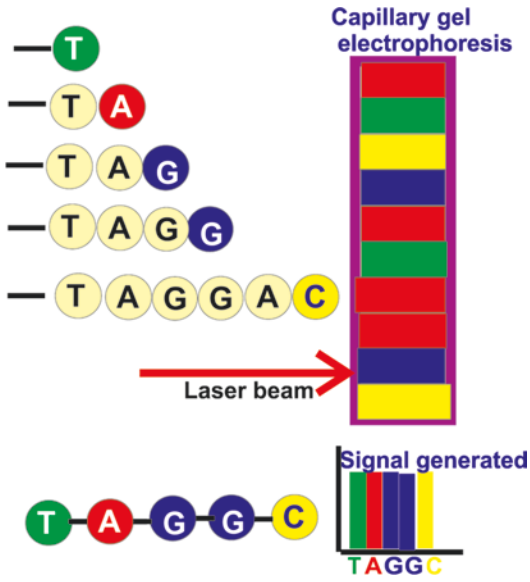

Fig. 23.2 Schematic diagram showing the capillary gel electrophoresis of different fragments of PCR products

23.2 Next-Generation Sequencing

Next-generation sequencing (NGS) is the newer gene sequencing technology that rapidly performs sequencing from abundant small fragments of DNA [2]. NGS is also called as high-throughput sequencing.

There are several categories of NGS:

1. Pyrosequencing
2. Microelectrophoretic methods
3. Hybridization sequencing
4. Real-time observation of single molecules

1. *Pyrosequencing* [3]: The pyrosequencing technology was the first available NGS system in the market. Pyrosequencing works on the principle of "sequencing of sequencing". In this technique fixed amount of inorganic pyrophosphate (PPi) is released whenever a nucleotide is incorporated in polymerization reaction by DNA polymerase enzyme (Fig. 23.3). The released PPi initiates a chain of reaction that liberates light energy and is detected by colour-charged device camera. The DNA sequence is assessed from the pyrogram that is generated during each nucleotide incorporation.

Advantages:

- Compared to Sanger sequencing, it is very fast.
- Much cheaper than Sanger sequencing.
- DNA sequencing of the different samples can be determined by the same run.

Limitation:

- Error-prone in case of the sequencing of long homopolymers

2. *Microelectrophoretic method*: In microelectrophoretic method microfabricated technical device is used for the electrophoretic separation of the DNA fragments [4]. Here an intricate ultradense channel arrays are made that help in micron precision. This technique has

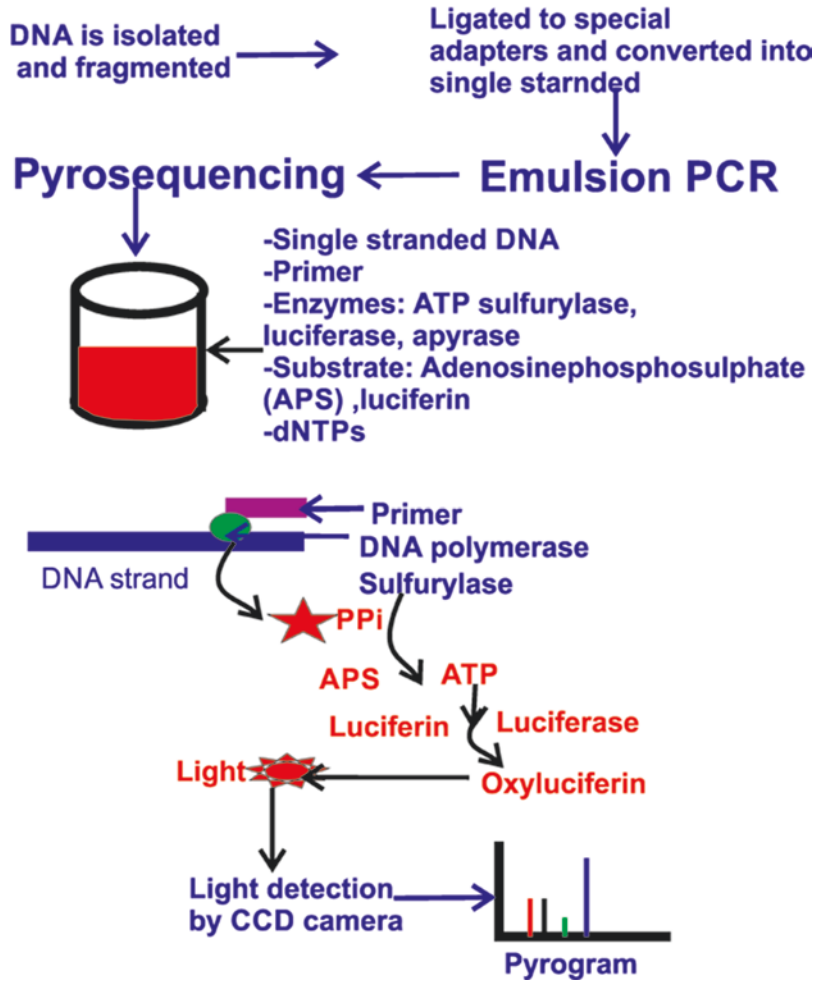

Fig. 23.3 Schematic diagram showing the basic principle of pyrosequencing technique. Here fixed amount of inorganic pyrophosphate (PPi) is released whenever a nucleotide is incorporated in polymerization reaction by DNA polymerase enzyme. The released PPi initiates a chain of reaction that liberates light energy and is detected by colour-charged device camera. The DNA sequence is assessed from the pyrogram that is generated during each nucleotide incorporation

integrated sample processing, DNA amplification, isolation and concentration of DNA fragments and sequencing.

Advantages:

- Very fast technique
- Minimal reagent consumption
- Good quality optical property

3. *Hybridization sequencing* (Fig. 23.4): In this technique, numerous oligonucleotide probes are used to hybridize with the target DNA. The complementary probes are hybridized with DNA, and therefore the sequence of bases of DNA is obtained. Finally the complete DNA sequence is decided by assembling the overall information from the multiple hybridization tests.

There are two ways to hybridize: (a) The DNA is immobilized on a membrane, and then various small oligonucleotide probes are used for hybridization. (b) Microfabricated tilling array contains more than 6,000,000 distinct probes, and the genomic DNA to be sequenced is hybridized to determine the complete base sequence of the entire DNA [5].

Advantages:

- Fast
- High throughput

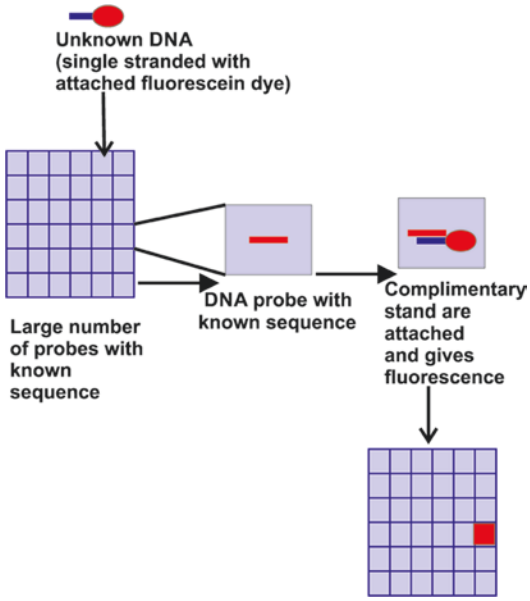

Fig. 23.4 Schematic diagram showing the basic principle of hybridization sequencing. In this technique, numerous oligonucleotide probes are used to hybridize with the target DNA, and the sequence of bases of DNA is obtained. Finally the complete DNA sequence is obtained by assembling the overall information from the multiple hybridization tests

4. *Real-time observation of single molecules*: In real-time sequencing, DNA chain is passed through a nanopore. This is an alpha-haemolysin pore covalently bound with cyclodextrin. The different base pair of DNA generates fluctuation of electrical signal during the passage through the nanopore, and the exact DNA sequence is assessed by measuring the fluctuation of pore conductance [6].

Advantages:

- There is no need of DNA amplification or cloning.
- Read length of the technique is much longer than other techniques.

23.2.1 Scope of NGS

- *Identifies broader spectrum of mutational changes*: In addition to base sequence of DNA, NGS detects all novel mutational changes such as small insertions and deletions.
- *Clinical decision support*: The demonstration of complete mutation data of a disease may help in the identification of the driver mutation. Once the driver mutation is identified, we can plan a therapy of the individual patient on the basis of it such as erlotinib EGFR mutation and vemurafenib in BRAF mutational melanoma.
- *Study of cancer genome for diagnosis, classification and prognosis*: NGS is able to provide the complete data of the individual cancers. This data may be helpful in the diagnosis, classification and prognosis aspects of the disease.

23.2.2 Limitations

- *Cost*: NGS is very costly and needs adequate infrastructure, experience, storage capacity and computational capacity.
- *Intratumor heterogeneity*: There may be variation of mutations in a particular type of tumor in different patients (intertumoral heterogeneity) or different mutational changes in a same tumor at different sites (intratumor heterogeneity). This is a complicated issue, and therefore the response of therapy may vary in a particular tumor.

23.3 Comparison of Sanger Sequencing and NGS

The differences of the above two techniques are highlighted in Table 23.1.

Table 23.1 Comparison of Sanger sequencing and Next-generation sequencing

Features	Sanger sequencing	Next-generation sequencing
Speed	Very slow	Fast
Knowledge of complete genomic data	The prior knowledge of the loci of the gene is needed	Unselective and no need of knowledge of loci of gene
Detailed molecular mutational change	Only base sequences possible	Detailed mutational changes can be demonstrated

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Part IV

Microscopy, Quality Control and Laboratory Organization

Compound Light Microscope and Other Different Microscopes

24

24.1 Light

Light is an electromagnetic wave with specific amplitude and wavelength. The distance between the two successive peaks of the light wave indicates the wavelength of the light. The distance between the crest to the horizontal axis is known as amplitude. The time to travel one crest to successive next crest is known as period. The frequency of light wave is indicated by the number of the wave cycle per unit of time (Fig. 24.1). The visible light remains within the wavelength of 400–750 nm. This is a very tiny fraction of the electromagnetic spectrum (the radio waves are 10^4 m and ɣ rays 10^{-10} m). The wavelength of the red light is 750 nm (1 nm = 10^{-9} m) and that of blue light is 400 nm (Fig. 24.2). Ordinary white light is comprised of all the colours. The dominant wavelength of the source of the light determines its colour.

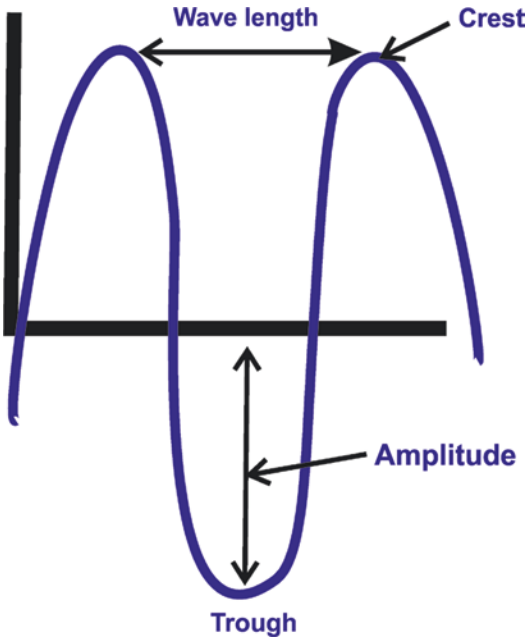

Fig. 24.1 Schematic diagram showing wave of light. The distance between the two successive peaks of the light wave indicates the wavelength of the light

24.2 Colours

There are different modules to express the colour.

Hue Saturation Intensity (HSI) This is one of the common modules to express colour. Hue indicates the basic colour such as blue, green or red. The saturation means how deep is the colour that means whether the colour is pale or dark. Intensity measures the brightness of the colour.

Red Green Blue (RGB) There are three primary colours: red, green and blue. The other colours that we perceive are the mixture of these colours such as admixture of red and green colour will produce yellow colour. The percentage

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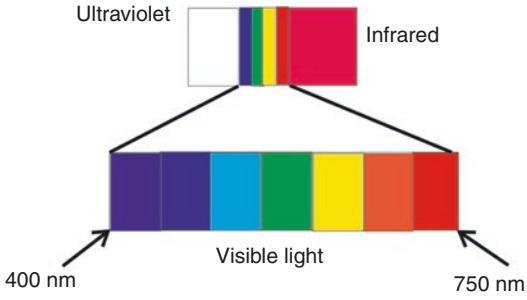

Fig. 24.2 Visible spectrum of light is 400–750 nm wavelength of light

expression of these three primary colours represents the colour of that particular object.

CYM The subtraction of one or more primary colour from the white light produces three other secondary colours: cyan (C), yellow (Y) and magenta (M). Therefore this CYM colour model is known as primary subtractive colours. This model is mainly used in optical filter and printing.

Perception of Colour Cone cells are the photoreceptor cells of the retina. They are exclusively related with the colour perception and consist of only 5% cells. The cone cells are mostly concentrated in the fovea centralis of the retina. We perceive white colour when the light equally stimulates all the three types of cone cells that means red, green and blue. If the light stimulates only the red-sensitive pigments of the cone cells, then we perceive red colour. Similarly if the light stimulates both red and blue pigments of the cone cells, then we perceive magenta colour.

24.3 Image Generation and Human Vision

Eye Is a Biological Camera The image of the object is formed on the retina of the eye (Fig. 24.3). At first the light rays from the object pass through the cornea, aqueous humour, lens and vitreous humour, and finally the light stimulates the photoreceptor cells of the retina. The initial image in the retina is an inverted image of the object. The photoreceptor cells of the retina pass the electrical signal through the optic nerve to the brain. Human brain corrects the inverted image to the normal erect image.

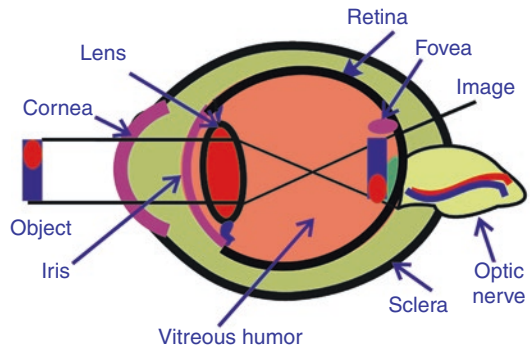

Fig. 24.3 Image formation in the human eye is highlighted in this schematic diagram. The image of the object is formed on the retina of the eye. The initial image in the retina is an inverted image of the object. The photoreceptor cells of the retina pass the electrical signal through the optic nerve to the brain. The human brain corrects the inverted image to the normal erect image

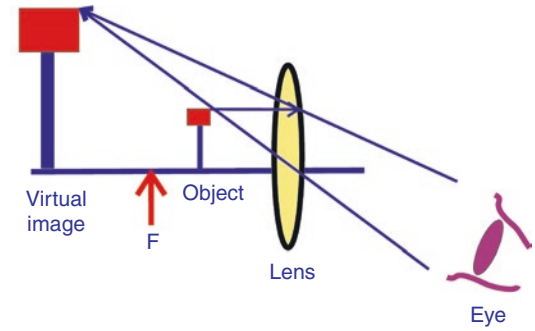

Fig. 24.4 Schematic diagram showing the formation of image by a convex lens. If the object is placed within the focal length of the lens, then we see a virtual enlarged image formed in the same side of the lens

Image Formation by Simple Lens The image formed in the retina of the human eye is non-magnified. The image of the object can be magnified if a convex lens is placed between the object and eye and appropriately focused (Fig. 24.4). If the object is placed within the focal length of the lens, then we see a virtual enlarged image formed in the same side of the lens. The focal length of the lens is defined as the distance between the optical centre of the

lens and the focal centre where the image is perfectly focused.

Light Microscope [1] The light microscope deals with the visible light and so it is named as light microscope. The predominate three functions of this microscope are:

- Magnification
- Resolution
- Contrast

Magnification The word magnification indicates the enlargement of the image of object of interest. The objective and the eyepiece take part in the magnification of the image of the object. The first magnification takes place by the objective. The power of magnification is written on the wall of the objectives of the microscope. Normally the power of magnification varies from 4 times to 100 times in ordinary biological light microscope. The second magnification is done by the eyepiece, and so the final magnification is equal to the first magnification done by objective multiplied by the second magnification done by the eyepiece.

$$M = M_1 \times M_2$$

M = Final magnification
M_1 = Linear magnification by objectives
M_2 = Linear magnification by eyepiece

The final magnification of the different objectives is shown in Table 24.1.

Resolution The term resolution means the ability of the microscope to distinguish two closely spaced objects. If we put two objects at a distance, then we are immediately able to identify them as two separate entities. However, the more and more they are kept close together, the more it is difficult to recognize them as two separate entities. At a certain distance, it may be impossible to distinguish these two objects, and they may look as one object. The minimum distance where the two objects can be distinguished separately is known as resolution capacity (D) of the microscope. The resolution of the microscope is dependent on two factors:

1. The wavelength of the light
2. The maximum angle of light that can be obtained by the objective lens from the object

Numerical Aperture (Fig. 24.5) Numerical aperture (NA) is related with the light gathering power of the objective. In fact the resolution power of the microscope is largely dependent on the NA of the objective, and it is represented by the equation below:

$$D = \frac{0.61\sigma}{\text{NA}}$$

D = resolution
Sigma (σ) = the wavelength of light
NA = numerical aperture
Numerical aperture is calculated as:
NA = n × Sin μ
n = refractive index of air (it is 1)
μ = half of the angular aperture of the objective

Table 24.1 Magnification of the microscope using different objectives

Objectives	Eyepiece	Final magnification
2×	10×	20×
4×	10×	40×
10×	10×	100×
20×	10×	200×
40×	10×	400×
100×	10×	1000×

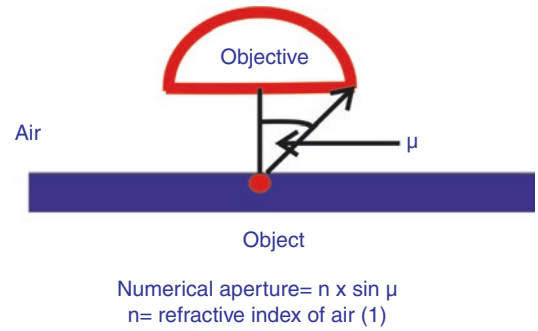

Fig. 24.5 Numerical aperture of the microscope is calculated as mentioned in the diagram. Numerical aperture is related with the light gathering power of the objective

Contrast The contrast of the microscope means the ability to detect an object from the background material. If we want to know the details of an object, then we need to have the difference of intensity of the colour between the object and the background material.

Image formation by the light microscope (Fig. 24.6)

The compound light microscope works in the same way as described in the magnification by convex lens (Box 24.1). The microscope is composed of bunches of properly placed lens that magnifies the image of the object in severalfolds. There are two sets of lens: objectives and the eyepiece. The object remains in between the condenser and the objective. The condenser condenses light through the object. The lens of the objective has short focal length and generates a magnified real image within the body tube of the microscope. Subsequently lens in the eyepiece further magnifies this real image, and a virtual more magnified image is formed approximately 25 cm distance from the eye in the same side of the lens near the object in the stage.

Box 24.1: Image Formation in Microscope

- First image is formed by the magnification by the objective.
- First image:
  - Real
  - Magnified away from the lens in the same side
  - Formed in the body of the microscope
- Second image is formed by the eyepiece which is the magnification of the first image.
- Second image:
  - Virtual
  - Highly magnified
  - Formed 25 cm away from the eye
  - Location: Between the stage and condenser

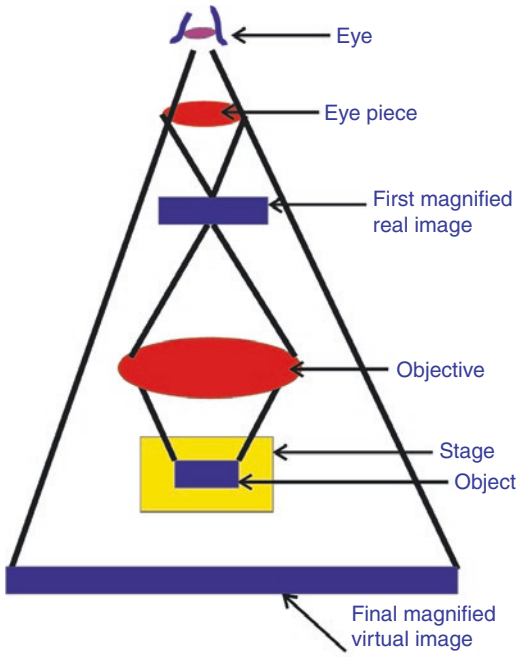

Fig. 24.6 Schematic diagram showing the formation of image by the light microscope. The lens of the objective has short focal length and generates a magnified real image within the body tube of the microscope. Subsequently lens in the eyepiece further magnifies this real image, and a virtual more magnified image is formed in the same side of the lens near the object in the stage

24.4 Anatomical Components of a Light Microscope (Fig. 24.7)

Base The base is the supporting platform of the microscope.

Stage The stage is the moveable component of the microscope. The slide is placed on the stage, and then the stage is moved up and down to focus the object. The slide on the stage is also moved in different directions.

Coarse and Fine Adjustment Knobs These are two knobs placed in both sides of the body of the microscope. These knobs are rotating and helping in the focusing of the objectives by moving it up and down.

Tube The tube is the connecting part between the objectives and eyepiece.

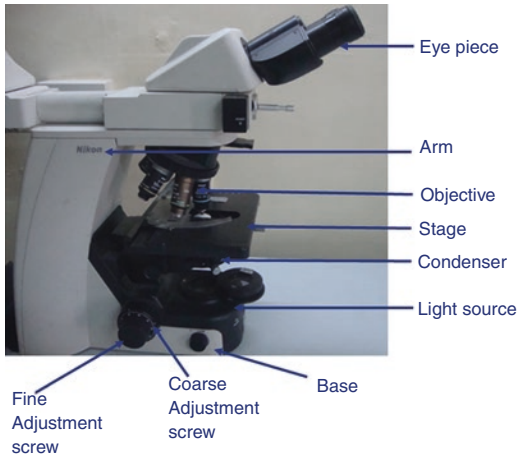

Fig. 24.7 Simple light microscope and its parts

Arm Arm connects the base of the microscope with the tube.

Revolving Nosepiece The various objectives are placed in the resolving nosepiece.

Diaphragm The diaphragm is located below the stage and helps to control the amount of light on the object.

Light Source The source of light is located on the base of the microscope; the light travels directly from the source to the object and then through different lenses.

24.5 Optical Components [2, 3]

The following parts are the constituents of the optical part of the light microscope:

1. *Condensers*: The condenser of the microscope accumulates light from its source and then concentrates the light on the object as a cone of light so that the maximum light passes through the object to get better resolution. The object is illuminated by parallel beam of light with uniform intensity. The condenser aperture diaphragm helps in controlling the diameter of the light beam. With the help of a screw, the condenser can be adjusted for the proper focusing of the cone of light. However once the objective is changed, then the position of the condenser has to be readjusted.
2. *Objectives*: Each microscope contains a set of multiple objectives attached with the rotating nosepiece. The circular ring of the objective represents the magnification power of objective, and the different objectives have different coloured rings on it (Fig. 24.8). The various information such as lateral magnification, numerical aperture, etc. are engraved on the objective. Presently the objectives are designed as infinitely corrected. The following are the different types of objectives:
   - *Achromat*: The achromatic objectives contain two doublets and a single front lens. They are relatively cheaper and quality suitable for the low magnification images. However they are not very satisfactory for higher magnification.
   - *Fluorite or semiapochromat lens*: Fluorite type of objective lens is made of fluorspar or newer synthetic substitutes and provides good level of colour correction. They are highly transparent and give high contrast. Therefore this type of objectives is suitable for immunofluorescence microscopy and polarized microscopy.
   - *Apochromatic*: These are relatively costly lens and give very good correction. The apochromatic objectives are the most suitable for colour photography as the chromatic aberration is nearly zero in such lens.

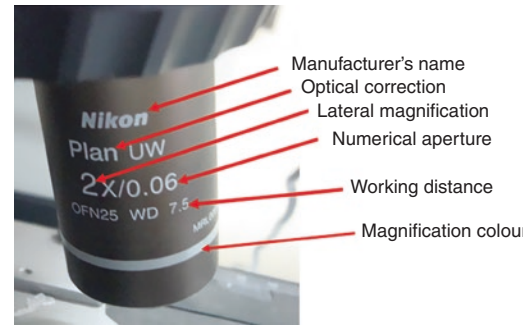

Fig. 24.8 Objective of the compound microscope. Note the basic information engraved on it

24.5.1 The Major Aberrations of the Lens

The spherical lens of the microscope may have several optical faults or aberrations. Image distortion occurs due to these various optical aberrations. The common aberrations are:

Chromatic Aberration (Fig. 24.9a) The chromatic aberration occurs due to the different refractions of different wavelengths of light. Blue light is bended more than the red light. Therefore there are different locations of images for the different wavelengths of the colour, and overall the image becomes distorted. More disastrous is the different magnifications of the different colours of images as they are located in different distances from the lens.

Solution: The solution of the chromatic aberration is to use the different components of the lens with different dispersing properties for the different colours so that the final images overlap in the same location.

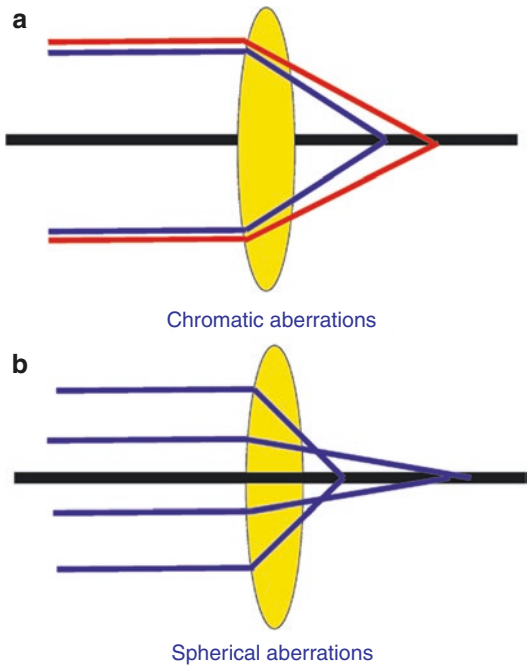

Fig. 24.9 (**a**) Principle of chromatic aberrations is explained in this schematic diagram. The chromatic aberration occurs due to the different refractions of different wavelengths of light. (**b**) Principle of spherical aberrations is explained in this schematic diagram. This is due to different bending capacities of the central and peripheral part of the spherical lens

Spherical Aberration (Fig. 24.9b) The spherical aberrations occur when spherical lens is used for magnification in the microscope. The cause of spherical aberrations is the different bending capacities of the central and peripheral part of the spherical lens. The incident rays of light that pass through the peripheral part of lens bend more than the incident rays that pass through the centre of the lens. Therefore in case of spherical aberration, the images are formed in different focal planes, and the image may be blurred in the periphery.

Solution: The solution of spherical aberrations is to apply a collection of different thickness of positive and negative lens.

Astigmatism This is an off-axis aberration, and it occurs due to the defect in the manufacture of the proper curvature of the lens or defective placement of the lens. Here the rays of light from the object going through the horizontal and vertical diameters of the lens focus in two separate focal planes. The astigmatism increases when the object is more away from the optic axis.

Solution: Good quality lens and proper placement of the lens

Curvature of Field In this aberration the image is curved instead of flat, and the whole image cannot be focused in a single flat plane.

Solution: The curvature of field can be corrected by proper designing of the lens of the objective.

3. *Eyepiece*: Eyepiece or the ocular lens magnifies the real image produced by the objective in the body tube of the microscope. Ocular lens generates the final image which is a virtual image and 25 cm away from the eye. The specification of the ocular lens is usually engraved on the lens barrel.

Nowadays most of the eyepieces provide magnification in the range of 10× to 20×. The ocular lens or eyepiece can be focusable or non-focusable. Eyepieces may be of two types:

Negative Eyepiece It contains internal diaphragm between the lenses of the ocularis (eyepiece). The simple negative eyepiece is also known as Huygenian eyepiece which is commonly used in routine light microscope.

Positive Eyepiece It contains internal diaphragm below the lens of the ocularis (eyepiece). This type of eyepiece is also called as Ramsden eyepiece.

24.6 How to Take Care and Handle Your Microscope

The basic care of microscope is highlighted in Box 24.2.

- *Starting*: Connect the plug of the microscope with recommended electrical power supply.
- *Focus*: Focus gently and do not be fast. During the focusing of the object, please take care so that the objectives do not touch the slide and break it.
- *Bulb:* The bulb of the microscope is costly so please turn off the illumination when the microscope is not used.
- *Transport*: Grasp the arm of microscope by one hand and support the base by the other hand. Keep the base of the microscope parallel to the ground so that the optical systems are not displaced or fall down. Never swing a microscope.
- *Touch*: Never touch the lens of the microscope by hand or any body parts because the normally producing oil in the body may blur the lens.
- *Cleaning*: Clean the lens of the microscope only by lens paper. Do not use any toilet paper or towel paper as they may scratch the lens. Do not use distilled water or any sorts of organic solvent as they may dissolve the coating of the lens. It is important to note that excessive cleaning may be harmful to the lens. The best way to clean a lens is using compressed air.
- *Closing the work*: After finishing the work with the microscope, please take out the slide from the stage, keep the low power objective towards the stage and far away from the stage, disconnect the electric power source and cover the microscope by dust cover.

Box 24.2: Basic Care of Microscope

- *Plugging*: Connect the plug in the recommended electrical power supply.
- *Bulb*: Always put off the bulb when you do not use the microscope. Remember that the bulb is expensive.
- *Focus*:
  - Do not hurry.
  - Gently focus your object and take care that the objective should not touch the slide.
  - Always use lowest power of objective first.
- *Cleaning*: Clean the lens before and after you use. Clean only by lens paper. *Do not use* distilled water or any sorts of organic solvent.
- *Transport*:
  - Grasp the arm of microscope.
  - Keep the microscope parallel to the ground.
  - Keep it close to your body.
- *Damage*: Never try to repair any damage. Report to the authority.
- *After finishing work*:
  - Unplug.
  - Do not put any slide on stage. Clean the stage.
  - Adjust the nosepiece for the lowest power objective.
  - Cover the microscope to protect it from dust.

24.7 Other Types of Microscope

24.7.1 Dark-Field Microscope

In this type of microscope, a hollow beam of light is produced by blocking the central part of the beam of light (Fig. 24.10). The scattered light from the object passes through the objective to the eye, and the object is seen as bright in a dark background. Ordinary light microscope can be used as dark-field microscope by using dark-field block condenser. The central cylinder of light is obstructed, whereas the peripheral rim of light reaches to the object.

Use:

- The bacteria, spirochaetes, or fungi in suspension are better seen in dark-field microscope.
- Movement of the cells in culture medium is better seen in this microscope.

24.7.2 Bright-Field Microscope

This is the simple light microscope where the object is examined by attenuated light. No additional equipment is required for the bright-field microscopy. The diaphragm of the microscope should be fully opened, and light intensity should be kept at low. The light passes through the sample, and the denser area of the object absorbs part of the light. So the object looked dark in a bright background.

Use: Stained or natural specimen

Advantages:
• Very simple.
• No additional equipment's are needed.
• Object can be seen without staining.
Limitations:
• Low contrast
• Low resolution
• Low magnification

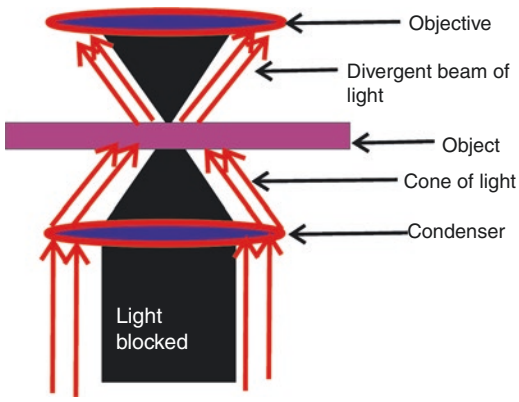

Fig. 24.10 Schematic diagram showing pathway of light in the dark-field microscope. A hollow beam of light is produced by blocking the central part of the beam of light

24.7.3 Phase Contrast Microscope

Principle In this microscope the objects having different refractive indices are identified as they produce different contrasts. The object with scattered light is identified from the illuminating background light. If the light passes through a transparent object, then due to the change of refractive index of the object, the pathway of light will be deviated slightly, and the light wave is retarded. In case of denser particle (higher refractive index) in the object, the deviation of light will be more, and the light wave is more retarded. This is known as phase difference. Usually these phase differences are invisible to us; however, the phase contrast microscope makes these changes significantly visible.

System (Fig. 24.11) In phase contrast microscope, a condenser annulus and modified objective with phase plate are used. Intense beam of light source is passed through the substaged condenser annulus which is located in the focal plane of the condenser. So a hollow cone of light is generated that can be controlled. Now this light passes through the objects/sample, and they are either deviated or undeviated depending on the refractive indices of the different structures of the object. Now both undeviated (central ray) and deviated light pass through the objective and are segregated by the phase plate that is located behind the focal plane of the objective.

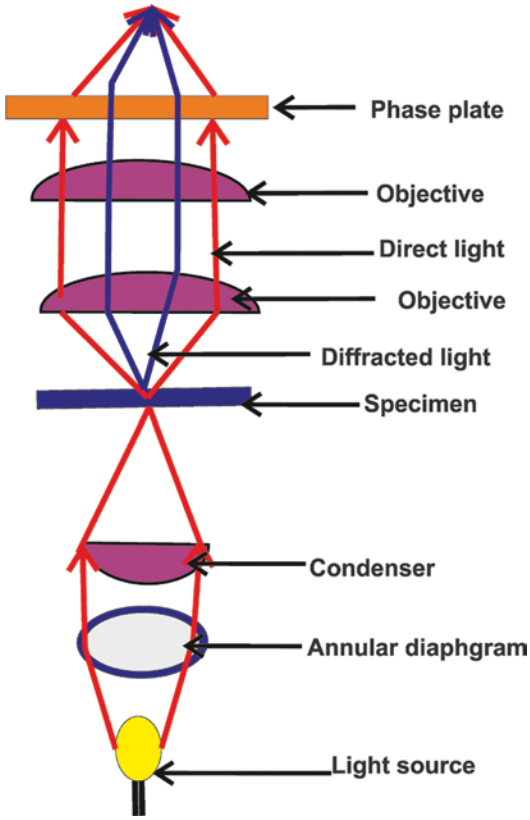

Fig. 24.11 Schematic diagram showing pathway of light in the phase contrast microscope. Here a condenser annulus and modified objective with phase plate are used. A hollow cone of light is generated that passes through the objects, and the lights are either deviated or undeviated depending on the refractive indices of the different structures of the object. The light passes through the objective and is further segregated by the phase plate behind the focal plane of the objective

Applications:

- This is used to see the living cells: shape, size, etc.
- Unstained protozoa or fungi are best seen in this microscope.
- Motility of the organism.

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25 Fluorescence and Confocal Microscope: Basic Principles and Applications in Pathology

Fluorescence microscopy applies high-intensity light to illuminate the substance that emits fluorescence light. The light of shorter wavelength strikes the fluorescent object, and the object emits the light of the longer wavelength, and the image of the object is visualized by the observer.

Principles of Fluorescence [1, 2] (Fig. 25.1) When a fluorescent molecule in the ground state absorbs a photon of excitation light, the electron of its outer shell jumps to the next orbit, and the molecule changes from the ground state energy level to the excitation state energy level. However within a fraction of second, very rapidly the molecule releases the photon of light and returns to the ground state again. In this whole process, the molecule losses some amount of energy and therefore vibrates in lower frequency. So the released fluorescent light has longer wavelength than the excitation light.

Basic Steps of Fluorescence Microscopy The basic steps of fluorescence microscopy are highlighted below:

- The high-energy light beam is passed through the filter and is directed to the object which is stained by a fluorescent dye.
- The fluorescent dye absorbs high-energy light and immediately releases photon of low-energy light.
- The emitted light is passed through the filter and is detected by the observer against a high-contrast black background.

25.1 Transmitted Fluorescent Microscope

In case of transmitted fluorescent microscope, we use mercury or xenon gas lamp for the high-energy excitation beam of light (Fig. 25.2). These burners contain gas with high pressure, and therefore careful handling is needed for these light sources. The beam of light generated by the burner passes through the heat-absorbing filter followed by the red light stop filter and wavelength selective filter. Now the beam of light hits the object, and the excitation beam passes through the objective towards the barrier filter. The barrier filter allows only the emitted fluorescence light to pass through it to the observer.

Selective Filter This type of filter specifically allows only the desired excitation beam of light. Careful selection of the filter is necessary for the selection of the wavelength of light nearer

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_25

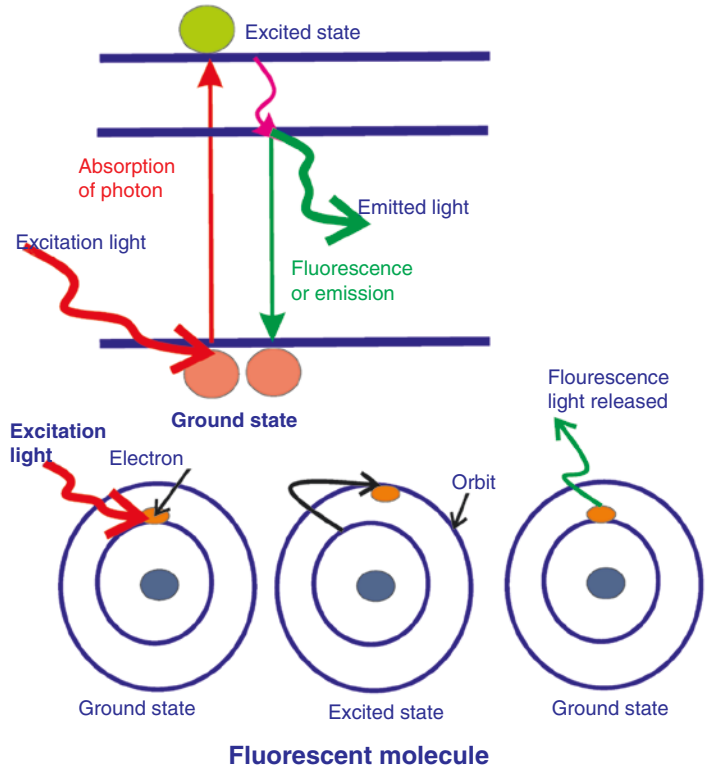

Fig. 25.1 Schematic diagram showing the principle of fluorescent dye

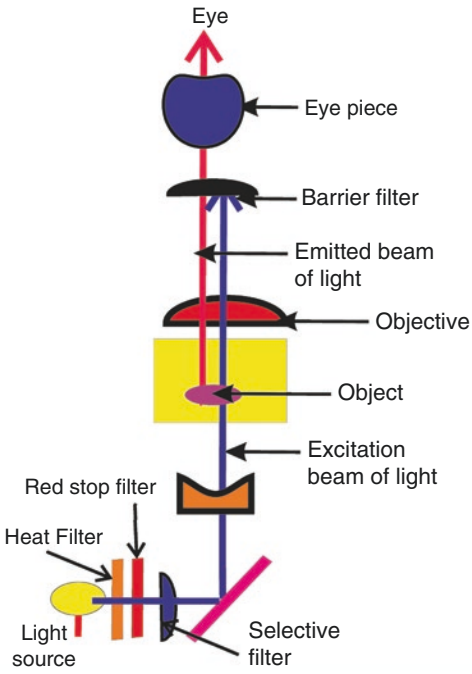

Fig. 25.2 Schematic diagram showing the principle of transmitted fluorescent microscope. The beam of light passes through the multiple filters and ultimately hits the object. The excitation beam passes through the objective towards the barrier filter and reaches to the observer

to the excitation maximum of the particular fluorescent dye.

Barrier Filter This filter is placed between the objective and the eyepiece. This type of filter prevents the passage of the light of short wavelength and helps to protect the retina. However barrier filter allows the emitted fluorescent light of longer wavelength.

25.2 Incident Fluorescent Microscope

In this type of microscope, a dichroic mirror is used (Fig. 25.3). This dichroic mirror has certain unique properties. The mirror only allows the selected excitation beam of light to be reflected on the object, and the remainder unwanted beam of light is travelled through the mirror and is lost. Similarly, the emitted fluorescent light from the object is allowed to pass through the mirror to the eyepiece. Therefore in incident fluorescent microscope, the high-energy light passes through the various filters, and then

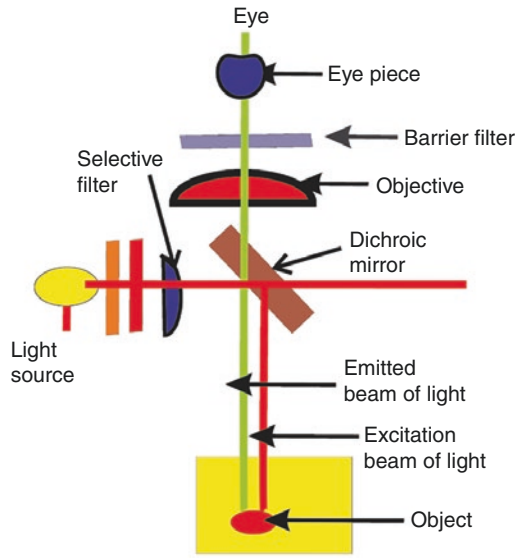

Fig. 25.3 Schematic diagram showing the principle of incident fluorescent microscope. In this type of microscope, a dichroic mirror is used that allows the selected excitation beam of light to be reflected on the object. Similarly, the emitted fluorescent light from the object is allowed to pass through the mirror to the eyepiece

with the help of a dichroic mirror, the selected wavelength of the light is allowed to pass through the objective to the object. The emitted fluorescent light from the object again passes through the dichroic mirror to the eyepiece. A barrier filter in between the objective and eyepiece is located and protects the eye from any high-energy light.

The advantages of incident fluorescent microscope:

- The dichroic mirror helps to gather most of the excitation beam and also reflects major part of emitted fluorescence. Therefore the image is much brighter in this type of microscope.
- Different types of objectives can be used in this microscope.

Dye used in fluorescence microscope

The common dyes used in fluorescence microscopy include**:**

- DAPI
- FITC
- Hoechst 33342
- Rhodamine
- Texas Red Cy3

Table 25.1 Maximum excitation and emission spectra of different fluorochrome dyes

Fluorochrome dye	Maximum excitation wavelength (nm)	Maximum emission wavelength (nm)
Fluorescein isothiocyanate (FITC)	494	520
Hoechst 33342	346	460
DAPI	359	461
Rhodamine	570	590
Cy3	548	561
Texas Red	595	613

These fluorochrome dyes are tagged with the specific antibodies or molecular probes. Each fluorochrome dye has a specific excitation spectrum that means the dye is excited by the light of specific wavelength which is also known as maximum excitation wavelength. Similarly the dye also emits photon of specific higher wavelength which is known as maximum emission wavelength. Table 25.1 shows the maximum excitation and emission spectra of different fluorochrome dyes.

Applications of fluorescence microscope

1. The fluorescence microscope helps to study the specific component of the cell or object of importance.
2. DNA and RNA sequencing.
3. To study the chromosomal abnormalities with the help of fluorescent in situ hybridization (FISH) technique.
4. Biomolecular assay.
5. Gene expression along with the location of the proteins in the living cell by green fluorescence protein (GFP).

25.3 Confocal Microscopy

The confocal microscopy (CFM) provides three-dimensional optical resolution. The principle of image formation in CFM is different than the

ordinary light microscope. In case of conventional light microscope when the lens (objective) is focused on a specific point of the object, the light comes from the entire depth of the object. Therefore in the fluorescence microscope, we get light from above or below the focal plane and the image becomes blurred. In contrast, in CFM at one time, we see the image of the particular depth of the object at a small point. All the out-of-focus light is eliminated by passing the light through the pinhole (Fig. 25.4). Multiple images at different depths are accumulated and then reconstructed to provide a three-dimensional image.

Principle (Fig. 25.5) In CFM the high-intensity laser light is directed to the object with the help of a dichroic mirror. The light hits the object, and the emitted fluorescence from the fluorochrome-stained object passes through the dichroic mirror to the confocal pinhole to the photomultiplier detector. The confocal pinhole aperture omits all the out-of-focus light from the upper and lower plane of the focus. It only allows to pass light that comes through the focal plane. Now multiple images from the different focal planes are collected by the computer software, and finally the three-dimensional image is constructed.

Components of Confocal Microscope
The major components of the CFM include:

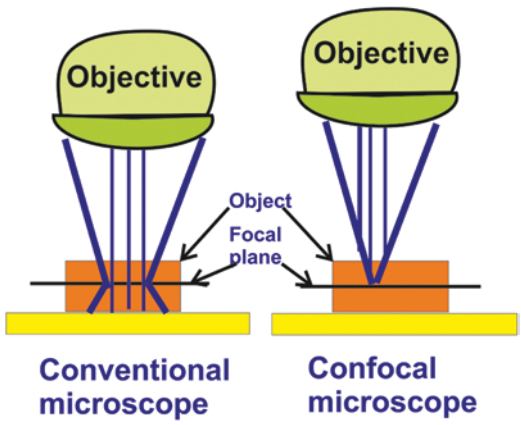

Fig. 25.4 Comparison of light scattering in conventional and confocal microscope. Light from the focal plane reaches to the observer in case of confocal microscope

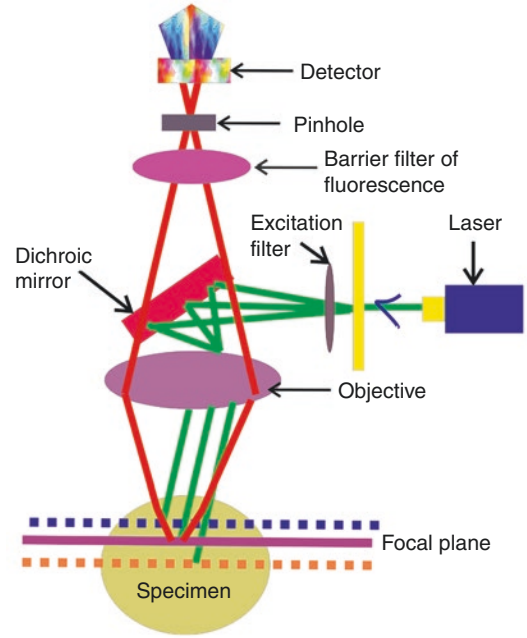

Fig. 25.5 Schematic diagram showing the principle of confocal microscope. The confocal pinhole aperture allows the light to pass that comes through the focal plane

1. *Light source*: Usually high power laser beam generated from laser or a mercury arc lamp.
2. *Microscope*: It has the X-Y axis controller along with Z axis stepper.
3. *Scan head*: This consists of a confocal aperture, shutter, scanning mirror and filter wheels. There are two types of scanning: (a) line scan occurs horizontally in X and Y axis and is usually very fast, and (b) frame scan occurs vertically in Z axis and is a slow scan process (Fig. 25.6). It helps to achieve the better temporal resolution. The confocal aperture is another important component of the scan head. There are two types of confocal aperture present: pinhole type and slit type. The different sizes of aperture are available, and the user can change them either manually or with the help of software. The barrier filter in the scan head prevents any unwanted light to allow to the detector (Fig. 25.5).
4. *Signal detector*: This is a photomultiplier tube (PMT) that converts the light energy into electrical energy. The light hits the phosphor and releases electron. The released electrons are

collected and stored as signal. The signals are finally displayed in a video monitor. There may be multiple PMT for the signal processing of simultaneously different fluorochrome dyes.

5. *Computer with appropriate software*: The computer is an essential component of CFM and has the following functions: image detection, image processing, image reconstruction, image storage and video display of the image.

Advantages (Box 25.1):

1. It helps to assess the spatial distribution of various intra- and extracellular macromolecules of the cells [3].
2. It provides high-resolution and high-contrast clear images.
3. CFM can work on living tissue, and the fixation of tissue is not required for the study of confocal fluorescence imaging.
4. Thick tissue section can be studied by CFM [4].

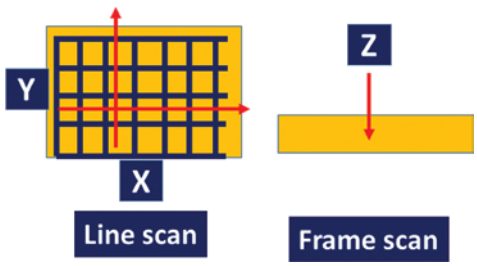

Fig. 25.6 Two types of scan occurs in confocal microscope: line scan and frame scan

Box 25.1: Advantages of Confocal Microscope

- Spatial distribution of intracellular and intercellular substances.
- High-contrast, high-resolution images.
- Live images possible.
- 3D reconstruction.
- Free from artefacts.
- Thick tissue can be studied.

5. Reconstruction of three-dimensional images is possible.
6. Images are free from artefacts seen in ordinary microscope.

25.4 Limitations of CFM

CFM has the following limitations:

1. Very expensive.
2. Photobleaching technique is not very good compared to conventional microscope.
3. Depth of penetration of CFM in live image is limited.

25.5 Applications of CFM [5–7]

Table 25.2 highlights the biological applications of CFM.

1. *Study of fluorochrome-stained section*: Single or multiple fluorescent dye-stained samples can be studied by CFM. It can detect simultaneously many fluorochrome dyes. Moreover fluorescence recovery after photobleaching (FRAP) is possible in CFM.
2. *The detection of co-localization*: CFM is helpful to determine the exact localization of two closely situated tissues. It provides blur-free very good resolution of fluorescent-stained sample.
3. *Green fluorescence protein (GFP)*: CFM can help to track the distribution and function of protein by tagging it with GFP. It helps in tracking cell signalling pathway, intracellular trafficking and also gene expression in the cells.
4. *Epitope tagging*: CFM is helpful in the tracking of epitope of the antigen by tagging it with green fluorescence protein (GFP).
5. *Diagnosis*: Screening of the colorectal cancer can be done with the help of CFM, etc. It also helps to measure the corneal thickening.
6. *Functions of cytoplasmic organelles*: Organelle-specific fluorescent probes are helpful to study the function of various cellu-

lar organelles such as mitochondria, endoplasmic reticulum, Golgi bodies, etc.

7. *Nucleus*: CFM helps to study the detailed spatial distribution of the different genes with the help of fluorescent in situ hybridization. It also helps to study the relative position of the chromosomal parts like telomere, kinetochores, etc.
8. *Morphometry*: Three-dimensional structure of the tissue can be studied by CFM. Even reconstruction of four-dimensional images (time considered as fourth dimension) is possible with the help of GFP.
9. *Microcirculation*: CFM helps in the assessment of blood circulation in small vessels such as velocity of the blood and also the distribution of various agents in the microvessels of the tissue.

Table 25.2 Applications of confocal microscopy in pathology

Area	Applications
Study of fluorochrome-stained section	• Possible to study single or multiple fluorescent dye-stained samples
	• Fluorescence recovery after photobleaching (FRAP) is possible
Co-localization	• Possible assess the exact localization of two closely situated tissues
Green fluorescence protein (GFP)	• Possible to track the cell signalling pathway, intracellular trafficking and also gene expression in the cells
Epitope tagging	• Possible to track the epitope of the antigen by GFP
Diagnosis	• Screening of the colorectal cancer
	• Measurement of corneal thickness
Functions of cytoplasmic organelles	• Organelle-specific fluorescent probes help to study the functions of cytoplasmic organelles
Nucleus	• Spatial distribution of the different genes
	• Relative position of the chromosomal parts
Morphometry	• Three-dimensional structure of the tissue
	• Four-dimensional images of the tissue
Microcirculation	• Velocity of the blood
	• Distribution of various agents in the microvessels

25.6 Two-Photon Microscopy

This is a three-dimensional imaging microscope. The basic principle of the two-photon microscopy is selective excitation of the fluorophore in a particular focal plane that means nonlinear excitation of the fluorophores. Unlike conventional fluorescence microscope, where single photon is absorbed by a fluorophore, in two-photon microscopy, two photons with half energy and double wavelength are used to excite the fluorophore (Fig. 25.7). The combined energy of two photons is optimum to excite the fluorophores. The excitation of the fluorophores by the two photons is the highest in the focal plane as the photon flux is maximum in the focal plane. The fluorophores above and below the focal plane are not excited. No pinhole is needed like CFM because the light from only that thin focal plane is emitted. In two-photon microscope, pulsed infrared laser beam is used to illuminate the object at a particular focal plane [8].

Advantages

1. Very good for live cell imaging as there is no photodamage of the living cell.
2. This microscope has the capability of resolution to several hundred microns and helps to study 1–0.5 μ thin section of tissue without any physical sectioning.
3. Better penetration of the infrared excitation beam of light.
4. The desired plane of section can be studied without wasting any tissue and therefore very effective in small biopsy material.
5. The microscope eliminates any contamination of fluorescence signal from up and down plane of the focus and therefore produces very good high-sensitivity image.

25.7 4Pi Microscopy

This is a specially equipped microscope that has the significantly improved capability of axial resolution. Increasing the angular aperture of the objective helps to increase the resolution of the

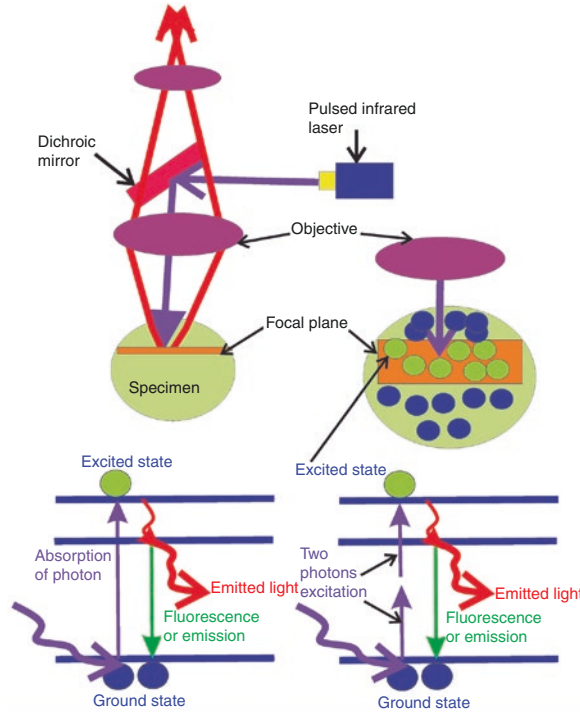

Fig. 25.7 The detailed principles of two-photon microscopy is explained in this schematic diagram. Instead of single photon, here two photons containing half of the energy are used. The combined energy of the two photons excites the fluorophore (see lower two figures). The laser beam can only excite the fluorophores in the focal plane (see upper left figure). The fluorescent light from the focal plane only selectively comes out

microscope. In 4Pi microscope the two identical objectives are used on both sides of the sample so that the effective angular aperture becomes two-fold [9] (Fig. 25.8). The increased angular aperture increases the resolution in the Z axis of the microscope (200 nm).

25.8 Spatially Modulated Illumination Microscopy

This type of microscope exploits the "Moiré effect". When two densely packed images are placed together, a new pattern develops. By manipulating the illumination, it is possible to reveal the exact image. The series of images with mildly changed illumination pattern are collected, and from these images with the help of computation, the underlying structure is revealed [10]. This spatially modulated illumination may be added in an epifluorescence microscope.

The Table 25.3 shows the comparison of different types of advanced microscopy.

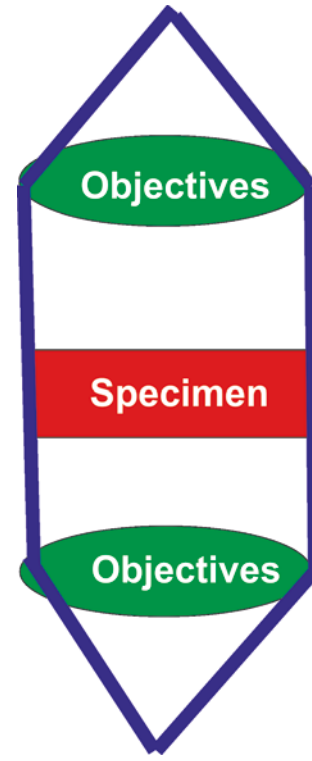

Fig. 25.8 Two identical objectives placed both sides of the sample in the case of 4Pi microscope

Table 25.3 Comparison of different types of advanced microscopy

Microscope	Principle	Advantages	Limitations
Confocal microscope	Pinhole is used to allow light from only a selected focal plane	Image of particular plane is seen, and three-dimensional reconstruction can be done	Limited depth of imaging is possible near about 100 μ
Two-photon microscopy	Two photons with half energy and double wavelength are used to excite the fluorophore and light from one particular focal plane	Optical sectioning of image is possible without any physical interference	Pulse laser is very costly
4Pi microscopy	Two identical objectives are used on both sides of the sample to increase the angular aperture of the objective and therefore to double the resolution in Z axis	Increased resolution of Z axis	It has limited diffraction
Spatially modulated illumination microscopy	The series of images with mildly changed illumination pattern are collected, and from these images, the exact image is computed	Add on to epifluorescence microscope	It has limited diffraction

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26 Electron Microscopy: Principle, Components, Optics and Specimen Processing

26.1 Introduction

Microscope magnifies the image of the object so that we can visualize the smallest particles. The resolution power of the light microscope is limited. The visible light has the wavelength of 300–700 nm. Light microscope uses the visible spectrum of light, and so the maximum resolution power of the light microscope is 0.2 μm (Fig. 26.1). The improvement of the resolution capacity of the microscope can only be improved by reducing the wavelength of the light. Ultraviolet ray has the wavelength of 100–300 nm and the resolution power is improved to 0.1 μm. Long-time scientists tried to find out the probe that has much smaller wavelength. During the first part of the twentieth century, the wave-like property of the electron was demonstrated, and subsequently this has been utilized in electron microscope (EM). The formula shows:

$$\lambda = \frac{h}{mv}$$

λ is wavelength, $h = 6.626 \times 10^{-34}$ (Planck's constant), m = mass and v = speed of the electron.

Now increasing the speed of the electron, we can reduce the wavelength significantly, and 0.001 nm wavelength of the electron can easily be achieved.

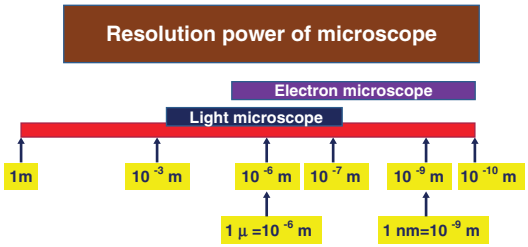

Fig. 26.1 Schematic diagram shows the resolution power of light and electron microscope

Therefore, with the help of electron as a probe, we can improve the resolution up to 0.1 nm (10^{-10} m).

The electron microscope is an advanced type of microscope [1, 2]. Unlike light microscope, EM is an expensive, large, fixed instrument and should be kept in a separate room. The differences between EM and light microscope are highlighted in Table 26.1.

EM uses high-energy electron beam to visualize the material under study [2]. The electron beam as a probe has several advantages.

1. Electrons have shorter wavelength and provide very high-resolution capacity.
2. It is easy to manipulate.
3. Electron gives high brightness.
4. Electron beam interacts strongly with matter

P. Dey, *Basic and Advanced Laboratory Techniques in Histopathology and Cytology*,
https://doi.org/10.1007/978-981-10-8252-8_26

Table 26.1 Comparison of electron microscope and light microscope

Characteristic features	Light microscope	Electron microscope
Probe used	Ordinary visible light: 700–300 nm	High-energy electron beam: 4 nm monochromatic
Maximum resolution	0.5 µm	0.1 nm
Maximum magnification	1000 times	500,000 times
Condenser	Made of glass	Electromagnetic coil
Objective	Made of glass	Electromagnetic coil
Interior of the optic column	Air filled	Vacuum
Image formation	On eye	On the fluorescent screen

26.1.1 Essential Components of Electron Microscope (Fig. 26.2a, b)

The main components of EM include:

1. Electron source
2. Sample illumination
3. Objective lens
4. Intermediate and projector lenses
5. DetectorsElectron Source (Fig. 26.3) Electron gun generates the beam of electrons. It consists of a:

(a) Tungsten filament
(b) Wehnelt cylinder (cathode shield)
(c) Anode plate

Tungsten filament is made of V-shaped tungsten wire. Alternatively lanthanum hexaboride crystal or field emission gun can be used for the source of electron. The V-shaped tungsten wire is encased with Wehnelt cylinder or cathode shield. The anode plate is located away from the cathode shield, and both the cathode shield aperture and anode plate are placed centrally in the same axis. Now a high voltage positive potential is applied to the anode plate, and simultaneously the tungsten wire is heated at 2700 K with the help of direct current. In this high temperature, the wire generates electrons by the process known as thermionic emission. The cathode shield is negatively charged and deflects the electron to make it a central beam. The central beam of electron emerges from the small hole of the (Wehnelt cylinder) cathode shield.

Sample Illumination (Condenser System) Several lenses are used as condenser for focusing the electron beam in a particular plane. Unlike light microscope, in case of electron microscope, we use electromagnetic coil as lenses. By applying electrical current through the coil, the strong magnetic field is created. The strength of the magnet can be changed by adjusting the electrical current through the coils. So if we increase the current, then the focal length of the beam will be shortened, whereas, reducing the current will increase the focal length.

Objective Lens The objective lens or imaging lens produces magnified image of the object. The objective lens has small focal length. The electrical current through the objective lens should be stable to have a highly focused stable image.

Intermediate and Projector Lenses Intermediate and projector lenses are used to change the magnification of the image further. The projector lens highly magnifies the last image created by the intermediate lens and focuses it on the screen or camera plate.

The Vacuum System The beam of electron should be in the vacuum chamber. The vacuum is needed because of:

1. The presence of gas molecule will collide with the electrons, and subsequently the gas molecule will scatter the electrons from their pathway. Therefore to maintain the optical pathway of the electron beam, the vacuum is mandatory.
2. The vacuum chamber prevents the oxidation of the tungsten molecule and therefore increases the longevity of the electron gun.

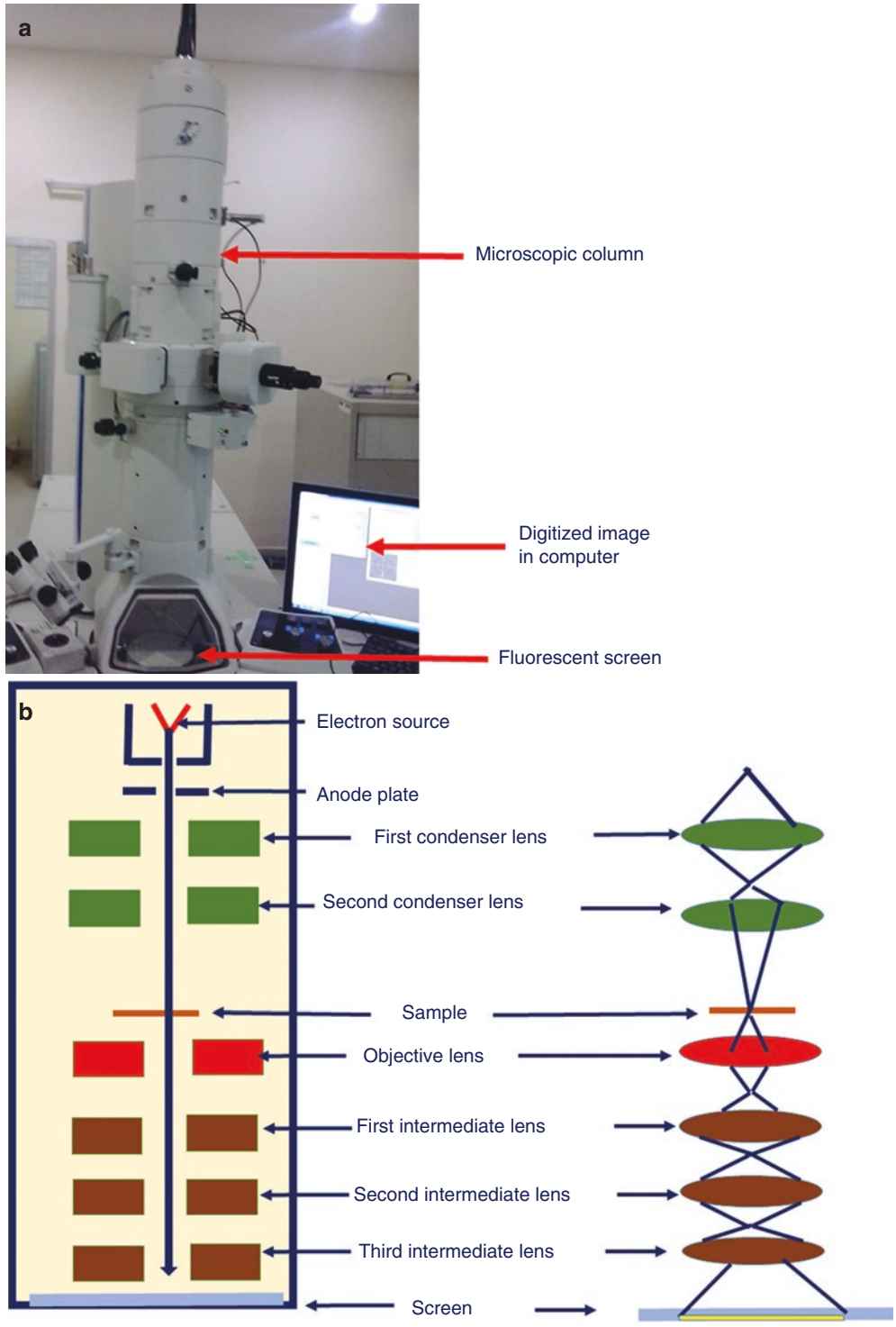

Fig. 26.2 (**a**) Electron microscope and its parts. (**b**) The various components of transmission electron microscope and microscopic column have been highlighted in this schematic diagram

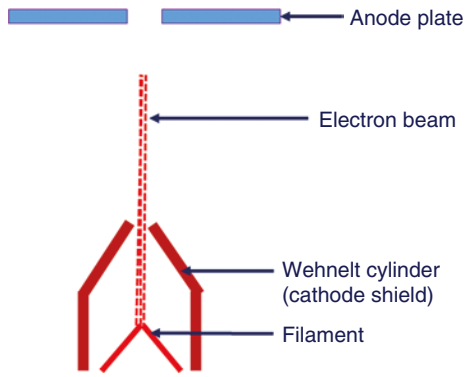

Fig. 26.3 The components of electron gun

To maintain the vacuum in the column of the microscope, multiple vacuum pumps are used at various levels. The maximum vacuum is needed between the electron source and the specimen. In most of the microscope, mechanical *rotary pump* is used that can generate vacuum of 1 Pa. This vacuum is still high for the tungsten wire electronic source. The diffusion pump can be added to obtain intense vacuum of 1×10^{-5} Pa. There is a chance of oil contamination in case of diffusion pump. Therefore to avoid this possibility, more expensive *turbomolecular pump* can be used which is a high-speed turbine fan. The *ion pump* is used in the modern EM equipment that can reduce the pressure up to 1×10^{-8} Pa. There are series of air locks in the system to isolate the parts of the column from the other sections. In the modern EM instrument, the vacuum chamber is automatically controlled by the electronic system.

26.1.2 Microscope Column and Electronic Optics (Fig. 26.2b)

The microscopic column has the following successive components:

1. *Electron source*: The high-energy electron beam is generated from the electron gun and is directed towards the condenser lenses.
2. *Condenser lenses*: There are two or more electromagnetic condenser lenses are present that subsequently focus the beam of electron on the sample. This helps in intense illumination in small area of the sample. As mentioned before we can change the focal plane of the condenser lens by adjusting the electric current.
3. *Sample*: The sample is placed in the microscope column below the condenser with the help of a holder that holds the grid containing the tissue section. Now the electrons interact with the thin tissue and hit the atoms of the tissue. Heavier atoms deflect the electrons and are known as electron-dense areas, whereas the electron passes through the lighter atoms and ultimately produces an "electron transparent" area. Therefore, we get a specific pattern of emerging electron beam.
4. *Objective*: The electromagnetic lens of the objective is very powerful and is the strongest lens in the whole system. It creates highly magnified image that is also known as intermediate image.
5. *Projector lenses*: The intermediate image is further modified and also magnified by the projector lenses. There are three sets of projector lenses:
   (a) Diffraction lens or first intermediate lens: It magnifies the first image created by the objective lens.
   (b) The second intermediate lens.
   (c) The third intermediate lens or final projector lens: This lens magnifies the image further and finally projects it on the screen of the detector.
6. *Detectors*: The final image is focused on the screen of the detector. This is a fluorescent screen, and when the electrons are bombarded on this screen, it emits light in the visible range to produce visible image. The image can be captured permanently with the help of a charge-coupled device camera.

26.2 Specimen and Electron Interaction

At the time of transmission of beam of electrons through the specimen, two types of interaction may occur (Fig. 26.4):

1. *Elastic scattering*: This is interaction between the nucleus of the atom and the electron of the beam (primary electron). In this type of reaction, the kinetic energy and velocity of the primary electron are unaltered. Only the pathway of electron is altered. The nucleus of the atom is very tiny (3×10^{-15} m) compared to the atom as a whole (3×10^{-10} m). Therefore, there is actually minimal chance of truly hitting the electron with the nucleus. However, the positive electrostatic force of the nucleus works on the electron and deflects it from its pathway.
2. *Non-elastic scattering*: Here, the principle electron of the microscope column interacts with the electrons of the orbit of the atom. The orbital electron repulses the incident principle electron. Here also the pathway of the electron is changed, and moreover the energy is lost by the principle electrons.

Secondary Electrons (Fig. 26.5) At the time of transmission of beam of incident electrons through the specimen, the incident electron may hit the atom of the specimen. The orbital electron gets excited and leaves the atomic orbit and moves towards the surface of the object. This released excited electron from the atom is known as secondary electron. The secondary electron also undergoes elastic and inelastic interaction and ultimately exits from the surface. This secondary electron can be detected. This is the basis of scanning electron microscope (SEM).

Backscattered Electrons When the high-energy incident beam of electron hits the specimen, some of the electrons of the incident beam are reflected back towards the surface. These electrons are known as backscattered electrons. The object with higher atomic number will have more backscattered electrons than that of lower atomic number objects.

Excited Electrons of the Atom The incident beam of electrons when hit by the electrons of the atom of the object, the atom changes in an excited state. This is due to the ejection of electron from the orbit of the atom. Later on, the atom comes to the stable unexcited state that occurs by shifting the electron from the outer shell to fill up the vacancy of the ejected electron. The excess energy is released in the form of Auger electrons, cathodoluminescence and X-ray.

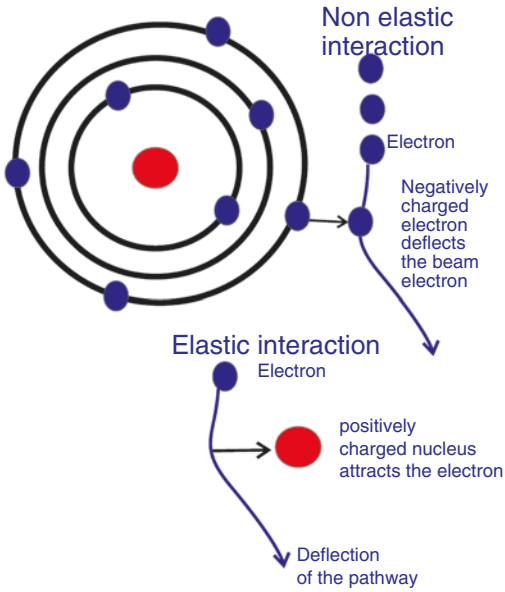

Fig. 26.4 Elastic and non-elastic interaction of electron. In elastic scattering the nucleus of the atom deflects the pathway of the primary electron of the beam. In non-elastic scattering, the principle electron of the microscope column is repulsed by the electrons of the orbit of the atom

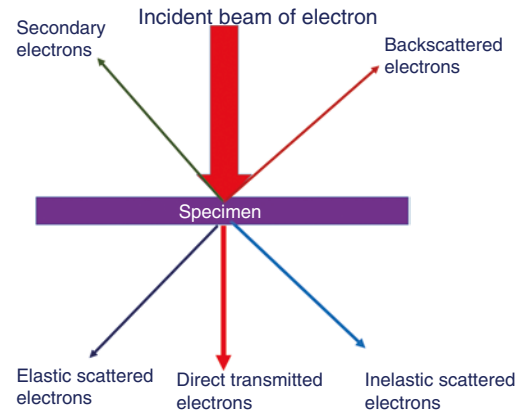

Fig. 26.5 Schematic diagram shows interaction between the electrons and the object. When the beam of incident electrons hits the majority of the electrons, they are transmitted through the object, and some amount of electrons is backscattered. Occasionally electrons from the object come out as secondary electrons

26.2.1 Electron Interaction in Transmission Electron Microscope

In case of transmission electron microscope (TEM), three types of interaction take place:

- The beam of incident electrons of the microscope column passes through the sample without any alteration of its path. The more thin the specimen, more amount of un-scattered electron will be transmitted. So this area will be lighter in colour, and the thick area will have less transmitted electrons and will appear darker in screen.
- A part of incident electrons will hit the nucleus of the atom, and elastic scattering will occur without any loss of energy. These scattered electrons will also pass through the specimen. The heavier atoms with more atomic number will scatter the incident electrons more than the lighter atoms with less atomic number. Same type of atom will form same type of scattered electron pattern.
- Lastly, another set of electrons will interact with the electrons of the atomic shell or orbit, and inelastic interaction will occur. These electrons will lose their energy considerably.

26.3 Sample Preparation for TEM

The preparation of sample is an important prerequisite for EM. The major criteria of the good sample preparation include:

1. The sample should be thin and electron transparent. The thickness of the sample varies from 30–50 nm, and the upper limit of thickness is 100 nm.
2. The sample should be mechanically robust so that it can withstand the handling in high temperature.

The steps of sample preparation for TEM include [3]:

1. Sample collection
2. Sample fixation
3. Dehydration
4. Clearing
5. Embedding
6. Sectioning
7. Staining

26.3.1 Sample Collection

The sample should be cut in small pieces of 1–3 mm in thickness of 1 square mm area. Try to fix the sample immediately. Transfer the needle biopsy sample directly into the fixative solution.

26.3.2 Fixation

The major aims of fixation are:

1. To prevent any change in the tissue and preserve the tissue as much as possible to its living condition
2. To prepare the tissue for the further processing so that the tissue does not disintegrate or tear

There is no ideal fixative for EM, and the choice of fixative depends on the type of tissue and the particular chemical constituents to study. The most commonly used fixative in EM is glutaraldehyde. However, glutaraldehyde alone is not suitable as the lipid is not fixed by it. Therefore, the best fixative is the combination of glutaraldehyde followed by osmium tetroxide.

Volume of Fixative The volume of fixative should be 15 times more than the volume of the sample.

Duration The average time of fixation is 9 h by 4% glutaraldehyde at room temperature and 1 h for osmium tetroxide. The tissue should not be in fixative for more than 12 h. Prolonged fixation is not recommended as this may extract the proteinaceous material from the tissue, and the proper sectioning will be difficult. Under fixation may cause swelling of the mitochondria and disruption of the other cell organelles.

Glutaraldehyde Fixation Glutaraldehyde causes cross-linking of the protein and denatures them. It stabilizes the protein without any coagulation. However glutaraldehyde is not a good fixative for lipids and causes cell shrinkage. This effect of glutaraldehyde can be balanced by osmium tetroxide which is a good fixative for lipid and causes swelling of the cytoplasm and nucleus.

Preparation

2% glutaraldehyde solution.

1M phosphate buffer solution

1M of sodium dihydrogen phosphate (NaH_2PO_4): 31.6 ml.

1M of disodium hydrogen phosphate (Na_2HPO_4): 68.4 ml of 1M.

Double-distilled water: 900 ml.

Maintain pH: 7.2.

Glutaraldehyde is available as 50% solution in 10 ml vial.

Now add 10 ml glutaraldehyde in 240 ml phosphate buffer solution to make 250 ml total solution.

Osmium tetroxide solution (1%)

Osmium tetroxide	1 gm
Distilled water	100 ml

Combined Fixation Technique *At first the tissue is kept in 2%* glutaraldehyde solution for 2 h. After 2 h the fixative should be poured out, and the tissue is washed in phosphate buffer solution for 5 min three times. Then the tissue is fixed in 1% osmium tetroxide for 1 h followed by two to three washing in double-distilled water.

26.3.3 Dehydration

Removal of water (dehydration) from the sample is necessary because most of the embedding media are not miscible with water. Therefore a dehydrating agent is used that remove the water and then replaces the water with a different solution which is soluble in the embedding medium.

The dehydration is done by treating the sample in the series of graded alcohol:

30% ethyl alcohol: 10 min

50% ethyl alcohol: 10 min

70% ethyl alcohol: 10 min

90% ethyl alcohol: 10 min

100% ethyl alcohol: 10 min

26.3.4 Embedding

The embedding medium helps to provide firm base for sectioning of the tissue and also to help in electron microscopy procedure. The ideal embedding medium should have the following desirable criteria:

1. Easy to cut the section
2. Stable in electron beam and withstand higher temperature (200 °C) at the time of microscopy
3. Easy to procure the medium
4. Evenly polymerized

Presently the following media are used for EM:

1. Epoxy resin
2. Acrylic media
3. Polyester resin

Epoxy Resin This is the most commonly used embedding medium for EM. The advantages of epoxy resin are:

- Stable at higher temperature
- Uniform polymerization
- No damage or tissue shrinkage

The main disadvantages of epoxy resin are high viscosity that requires long infiltration time. Epoxy resin also causes dermatitis and therefore any direct contact of this substance should be avoided.

The vial cap should be taken out and the tissue is embedded in freshly made resin for overnight at 60 °C.

Acrylic Media Butyl methacrylate and methyl methacrylate are the common acrylic media. They cause significant cell shrinkage (20%). At the time of polymerization, bubble formation may occur and this may damage the block.

Moreover methacrylate may undergo sublimation and disintegrate in the presence of high-energy electron beam.

Araldite: Araldite is an aromatic amine. This is one of the epoxy resins used for EM. Araldite is used in combination with a hardener, an amine accelerator and a plasticizer. The amine accelerator accelerates the reaction between the resins. The components should be mixed properly to avoid the formation of any air bubbles.

Epon: Epon is an alternative embedding medium for EM. This is an aliphatic resin and has low viscosity. Therefore it can infiltrate within the tissue more quickly compared to Araldite.

Polyester Resin Polyester resins have similar properties as that of epoxy resin. They do not cause any cell shrinkage and polymerize uniformly. Vestapol W is the commonly used polyester resin.

26.4 Sectioning

To make a thin section is the crucial point of sectioning. Ordinary histological microtomy is not suitable for the electron microscopy sectioning, and ultrathin microtomy is needed.

Knives *Glass knives*: Glass knives are cheap and convenient (Fig. 26.6).

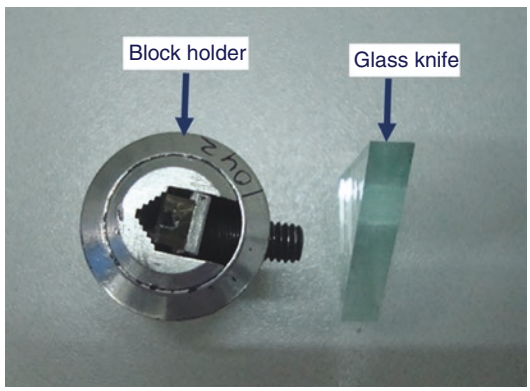

Fig. 26.6 Block holder and glass knife used in electron microscope

- *Diamond knives*: The diamond knives are relatively expensive. The section quality of diamond knives is far better than glass knives. These knives are more durable than the glass knives.

Semi-thin Sections It is the preliminary screening procedure to see the adequacy of the sample. At first the resin-embedded blocks are trimmed to expose the underlying tissue. Approximately 1 μ thick multiple sections are cut from each block. The sections are picked up from the water trough placed directly below the glass knife. The semi-thin sections are dried in a hot plate at 60 °C. The dried sections stick to the glass slide. The semi-thin sections are stained with 1% toluidine blue in 1% sodium tetraborate solution for 1 min to see the adequacy of the sample. If the section contains the representative areas, then further ultrathin sections are made from the block.

Toluidine blue 1% solution
Toluidine blue: 1 g
Sodium tetraborate: 1 g
Distilled water: 100 ml

At first dissolve the borax in the distilled water. Then add toluidine blue and dissolve by constant stirring. Filter the solution and keep it at room temperature.

Ultrathin Section The trimmed blocks are cut further by an experienced technician. The ultramicrotome is set in an auto mode to have optimum thin sections (Fig. 26.7). As mentioned before we need less than 100 nm thick section and the optimum thickness is 80 nm. The reflected light from the section gives information about the thickness of the slide:

Grey colour: <60 nm
Silver colour: 60–90 nm
Gold colour: 90–120 nm

The sections float either in ethanol or acetone. To stretch the sections, one can take the help of xylene or chloroform. A small piece of filter paper soaked with either xylene or chloroform can be hold just above the section. The evapo-

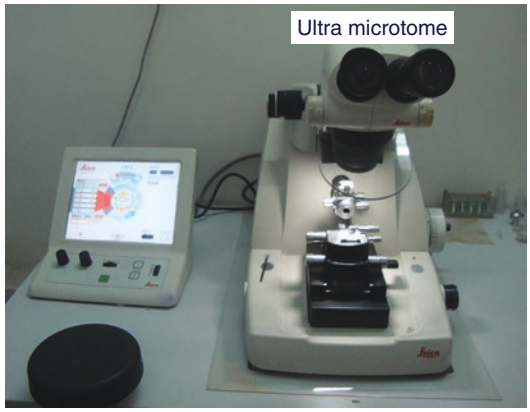

Fig. 26.7 Ultramicrotome used in electron microscope to cut ultrathin section

rated vapour usually stretches the section. The stretched sections are finally picked up by small copper grid. There are shiny and dull side of the copper grid. The sections are lifted on the dull side of the grid.

26.4.1 Staining of the Sections

The sections are stained along with grid. They are commonly stained with lead or uranyl acetate.

26.4.1.1 Lead stain

Reynold's lead citrate solution is used for the staining. Lead rapidly reacts with the atmospheric carbon dioxide and may form lead carbonate as precipitate. Therefore adequate care should be taken to prevent such precipitation. The solution should always be filtered before use.

Stain:

- Stain the section by dipping it in Reynold's lead citrate solution for 15 min.
- Wash each grid by 0.1 N NaOH solution.
- Wash by two change of distilled water.
- Dry the grid and keep it in a grid box.

Reynold's Lead Citrate solution
Lead nitrate: 1.33 g
Sodium citrate: 1.76 g
Distilled water: 30 ml

Mix lead nitrate and sodium citrate in distilled water and shake them for 1 min.

Now add 8 ml 1M NaOH and mix them well. Gently add 50 ml distilled water to dissolve the precipitated lead nitrate. Keep pH 12. The solution will be stable for 6 months.

26.4.1.2 Uranyl Salt

Aqueous or alcoholic solution of uranyl acetate is used for the staining of TEM. Uranyl acetate combines with protein and lipids and gives good contrast of various membranes and nucleic acid. The major disadvantage of uranyl acetate is the rapid precipitation in the presence of light.

Either aqueous or alcoholic solution of uranyl acetate can be used. Alcoholic solution has short staining time, and it penetrates easily within the sample. It also gives better contrast. Saturated alcoholic solution of uranyl acetate is used for staining.

Aqueous solution of uranyl acetate also provides good contrast. However this solution is photosensitive and therefore rapidly precipitates.

The overall comparison of the laboratory procedure of light microscope and electron microscope is highlighted in Table 26.2:

Table 26.2 Comparison of processing, staining and sectioning between light microscope and electron microscope

Characteristics	Light microscope	Electron microscope
Fixative	10% formalin	2% glutaraldehyde (2%) and osmium tetroxide (0.1%)
Embedding medium	Wax	Epoxy resin
Section thickness	4–5 μ	80 nm
Cutting	Ordinary microtome	Ultramicrotome
Knife	Disposable knife	Glass or diamond knife
Section holding	Glass slide	Copper grid
Routine stain	Haematoxylin eosin stain	Lead impregnation

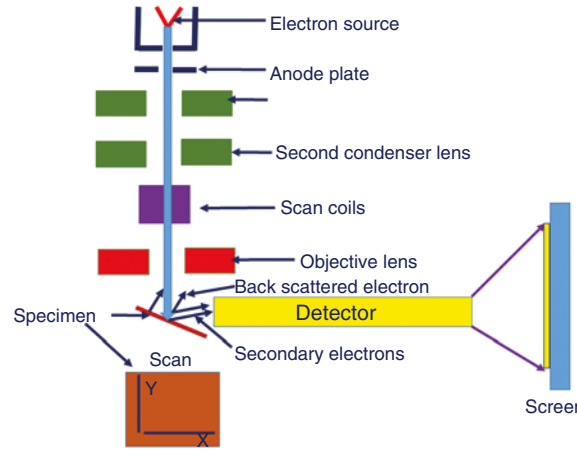

Fig. 26.8 Schematic diagram shows the various components of scanning electron microscope. Here instead of transmitted electrons, the secondary electrons and the backscattered electrons are recorded

26.5 Scanning Electron Microscopy [4]

Scanning electron microscope (SEM) provides information of the surface structures of the object. The spatial resolution of the SEM is ten times better than the light microscope. The image of the SEM is developed point by point from the emitted secondary electrons like a scanner image. Therefore the instrument is known as SEM.

26.5.1 Operational Principle

As mentioned before, when the electron beam hits the specimen, the secondary electron and backscattered electrons come back from the surface (Fig. 26.8). These electrons emitted from the same side of the incident beam create an image. Therefore the construction and operational mode of SEM and TEM are totally different (Table 26.3).

Specimen preparation for SEM: The fixation, processing, sectioning and staining for SEM are same as that of TEM.

Table 26.3 Comparison of transmission electron microscope and scanning electron microscope

Transmission electron microscope	Scanning electron microscope
Incident beam of electron is static	Incident beam of electron is dynamic and scans the object horizontally in two perpendicular directions
Image is formed instantly	Image is formed like a scanner image
Incident beam of electron passes through the object	Incident beam of electron hits the object and emits secondary and backscattered electrons that forms the image

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Quality Control and Laboratory Organization

27

27.1 Introduction

In the manufacturing company, we produce the final product from the raw materials and then deliver to the customers. The final product is always verified to maintain a particular standard. Such as a car company assembles the raw materials and after making a car, the company takes measure to maintain the standard of the car. If there is any defect, the company immediately rectifies it and also prevents to produce such defects in the future. In the histopathology (or cytology) laboratory, we receive the tissue or sample, process the sample, make stained section on the slide for the interpretation and finally report the tissue sample. This is very similar to the industrial company. Stringent maintenance of quality or standard is also needed for good laboratory service. Several terminologies arise in this area that are defined below.

Quality Control The term quality control (QC) means the collection of operational techniques to verify and maintain a desired set level of quality in the laboratory test or process [1]. The quality control activity is a continuous process, and it starts immediately from receiving the specimen to the final dispatch of the report along with post verification of the test result.

Quality Assurance Quality assurance (QA) is defined as the program that does systemic monitoring and evaluation of various areas of the quality control result and quality practice so that the laboratory delivers excellent healthcare service [2].

The QA is a co-ordinated dynamic process that detects the error and takes measures to control and prevent it in order to provide the best healthcare service.

Quality Improvement (QI) QI means an overall attempt to improve the specific quality of the laboratory by assessing the performance and improvement of the laboratory service.

Objectives of Quality Control The main objectives of the quality control are:

1. To give correct and complete test report to the patient
2. To generate and delivery the report in a minimum amount of time
3. To maintain ethics and professional service
4. To provide excellent service to the patient so that it satisfies the patient
5. To provide continuous training and current education to the laboratory staffs

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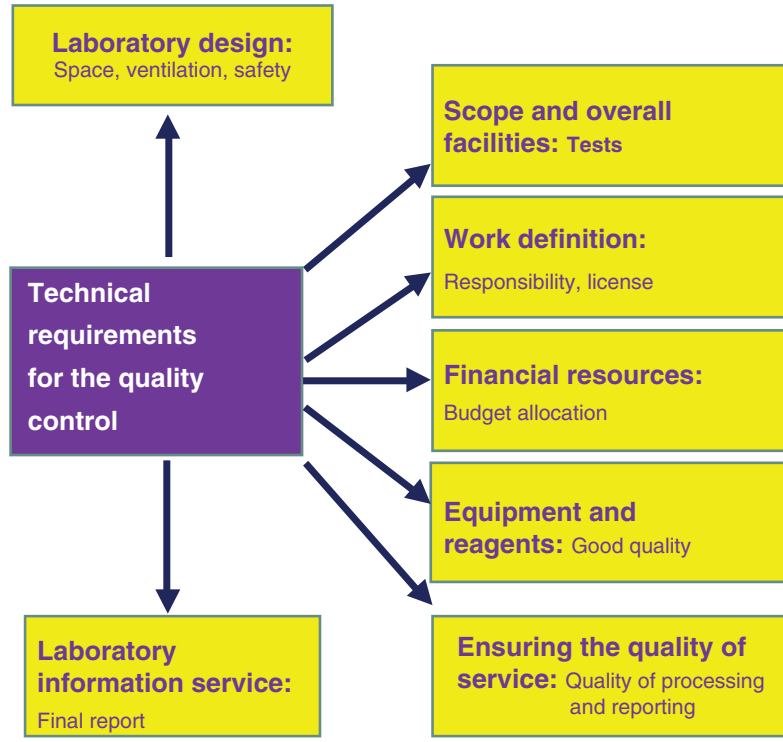

Fig. 27.1 Technical requirements for quality control in the laboratory are highlighted in this diagram

Essential Technical Requirements for Quality Control The essential technical requirements for the quality control measures include (Fig. 27.1):

1. *Laboratory design*: The laboratory should be designed in such a way that there remains enough space for receiving the sample, processing and staining and for the interpretation area, storage, etc. There should be proper ventilation and safety arrangement in the laboratory.
2. *Scope and overall facilities in the laboratory*: Overall laboratory facilities should be clearly documented. Detailed description of all the tests in the laboratory should be mentioned to the patients.
3. *The work definition of the laboratory personnel*: The work responsibilities of the different categories of the laboratory staffs should be clearly described. The staffs should be highly competent with professional licence to practise the respective work.
4. *Financial resources*: It is necessary to know the overall financial budget allocation for laboratory personnel, equipments, chemicals, etc. This knowledge of the financial budget gives the idea of the capability of the laboratory to fulfil the customer's need.
5. *Laboratory equipments and reagents*: The standard equipments and reagents are needed to provide good quality well-stained sections and smears. The microtome, processing machine, etc. should be regularly updated. There should be a proper log book mentioning the use of the equipments, purchase date and expiry date of the chemicals.
6. *Ensuring the quality of the processing and reporting*: The quality of the processing should be regularly checked and recorded. Similarly the reporting quality should be verified periodically.
7. *Laboratory information service (LIS)*: LIS generates unique accession number of the specimen. This number provides the identification of the sample or section. The patient's clinical history and other necessary information are listed in LIS. The final report is also entered in LIS, and the report is recoverable instantly by the end service providers.

27.2 Quality Control

Quality control involves three important steps (Fig. 27.2):

1. *Pre-analytic phase*: It starts from receiving the sample up to the final processing for reporting.
2. *Analytic phase*: It mainly involved the interpretation of the test or slide (in case of histopathology or cytology service).
3. *Post-analytic phase*: It is the post-interpretation phase and involves the report delivery, storage of slide, review of the slide or test, etc.

27.2.1 Pre-analytic Phase

The pre-analytic phase has the following components:

1. *Receiving the sample*: This is the first step of the quality control. The laboratory staff in the reception should follow the following aspects:
   - Identification of the sample and the patient's requisition form: The request form should always include the following information: name of the patient, name and address of the requesting consultant, test required, clinical history and diagnosis, drug history, etc. The sample and requisition form should be identified properly.
   - Allocation of the unique accession number to the patient's sample: This unique accession number of the sample should be allocated immediately after the initial registration of the patient. This can be given as a computer-generated bar code number and can be given in all the subsequent tissue, blocks and sections.
   - Patient's clinical history to include in the computer.
   - Check the proper fixative for the histopathology and cytology sample: The fixation of the specimen is a necessary step as delayed fixation may cause significant autolysis. Surgical specimen should be fixed immediately with 10% buffered formalin. Cytology sample should be sent in proper recommended fixative.

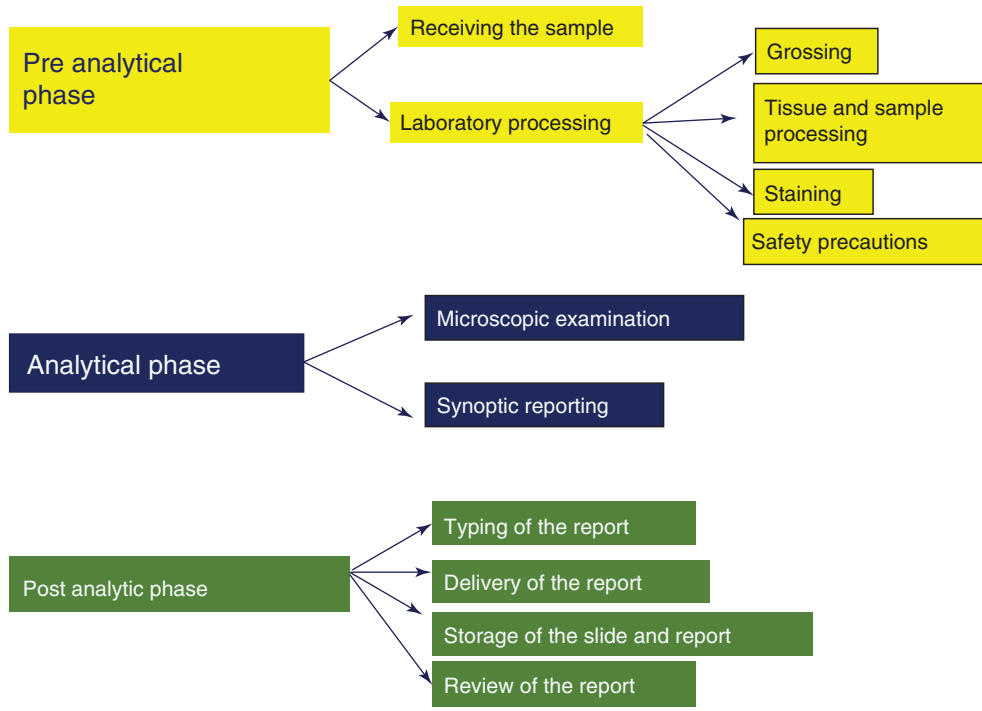

Fig. 27.2 Three important steps of quality control: pre-analytic, analytic and post-analytic phases

27.2.2 Laboratory Processing

- *Grossing*: Strictly speaking, grossing of the sample is an analytic phase. This is the duty of the qualified pathologist. The grossing room should be well equipped with gloves, knives, scissors, measuring tape and weighing machine. The automatic bar code accession number can be attached in the block. This bar code number is same as that of in the requisition form and the specimen.

27.2.3 Tissue and Sample Processing

(a) *Processing*: The tissue and sample should be processed according to the standard operating protocol (SOP) of the laboratory. The bar coding of the block and slides help to track the individual cases, and this prevent potential mix-up. The stained slides may be scanned by a whole slide image system. This may replace the glass slide in the future. Recently FDA has approved whole slide scanning image to report directly from the monitor bypassing the glass slide (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm552742.htm).
(b) *Vigilance by the senior laboratory personnel*: It is mandatory to check the section/smear quality periodically. The error should be mentioned in the log book along with the remedies taken to overcome the problems.
(c) Log book of equipments, reagents, etc. should be maintained.
(d) *Health and safety of the staff*: Laboratory supervisor should do close scrutiny and effective measures to maintain the safety and health of the laboratory staffs.

27.2.4 Analytic Phase

Microscopic Examination This is the vital step and the final interpretation of the slide is always done by a qualified licenced pathologist. In a histopathology laboratory, the preliminary and final interpretation are done by the junior and senior pathologists only. However, in a cytology part, the cervical smears are screened by the primary screener who may be a cytotechnologist. The final reporting should always be done by the consultant cytopathologist [3].

Synoptic Reporting Format The pathologist should follow the standard reporting format. Synoptic reporting is helpful to cover all the information regarding the sample particularly in a large specimen. In addition, the synoptic reporting format generates uniform reporting and is more efficient. The data analysis and research are easier in such format.

Cytology *Cervical smears*: After the initial screening by the cytotechnicians, re-screening is recommended to reduce the false-negative rate. Commonly used re-screening techniques are:

- *Proportional re-screening*: A certain fraction (10%) of the negative smears are screened by the cytopathologist.
- *Selected re-screening*: The cytopathologist re-screen only the selected high-risk cases such as previously diagnosed abnormal smears, history of bleeding per vagina, etc.
  - *Rapid review*: In this method all the smears are re-screened rapidly to pick up the false-negative cases.
  - *Automated re-screening*: The computer screens all the smears and indicates the smears to be re-screened again.

Number of slides to screen: The primary cytoscreener should not screen more than 100 slides in 1 day. The maximum time limit to screen the slide is 8 h [4].

Fine needle aspiration cytology smears and non-gynaecological smears:

- The cytopathologist should follow the consistent pattern of reporting and should discard the ambiguous terminologies as far as possible.
- In problematic cases the consultant should take the opinion of other fellow colleagues.

27.2.5 Post-analytic Phase

This phase enjoys the relative freedom from time pressure. The implementation of the quality assurance is applicable in the post-analytic phase. This phase involves:

- Proper typing of the report
- Manual or electronic delivery of the report
- Storage of the slide and report
- Review of the signed out report

Quality Check of the Signed Out Report The following measures may help in this aspect:

- Review the report of a specific system by the expert second consultant in that system.
- Random review of certain percentages of cases (2–10%) depending on the resource of the laboratory.
- Different interdepartmental meeting: Liver biopsy round, kidney biopsy round, various oncology meeting, clinicopathological conference, etc.
- Correlation of frozen section and permanent section.
- Cytology and final histopathology correlation: All the discorrelated cases should be discussed in detail so that the error can be overcome in future.
- Review of the cases by other institutions.

27.2.6 Gold Standard

Cytology Final histopathology report is the gold standard of the cytology cases.

Histopathology In case of histopathology cases, clinical follow-up of the patient is the ultimate gold standard. The opinion of the external expert may be taken into consideration as final judgement. In case of death, the final autopsy report should be considered as the gold standard.

Type of Errors The following types of error may occur [2]:

- Categorical change: benign versus malignant.
- Error in typing: type of malignancy is wrongly made.
- Error in classification.
- Error in the involvement of lymph node.
- Error in the interpretation of the margin of the resected tumour specimen.
- Mistake in the identification of side: right versus left.
- Error in the identification of the patient.

The Rectification of Error It is essential to correct the detected error. The new revised report may consist of:

- Corrected diagnosis.
- Corrected information.
- Any additional information should be given as footnote.

27.2.7 Record-Keeping

All the data should be stored preferably in the computer. There should be proper backup of the data.

Royal College of Pathologists, UK, recommends [5]:

- Preserve the tissue block forever.
- Preserve the histopathology slides for 10 years.
- Keep the wet tissue for 4 weeks after the dispatch of the report.

There are definite guidelines for storage of cervical smear and this are [6]:

- Irrespective of the diagnosis the cervical cytology smear should be kept for 5 years.
- The test requisition form should be retained for 2 years.
- Test reports must be retained for 10 years.

Interlaboratory Comparison The laboratory should join in the interlaboratory slide exchange program. The primary diagnosis offered by the laboratory should be verified by other groups of laboratory and vice versa. There should be a periodic meeting or feedback to correct the error.

27.3 External Quality Assurance

External quality assurance consists of:

- Proficiency test
- Continuing medical education

Proficiency Test The proficiency test is a voluntary program. The various laboratories should take part in the proficiency test to improve the diagnostic skill. In UK, the proficiency test is mandatory for the reporting consultants who work in the NHS Breast Screening Program. Overall the proficiency test is educational, and it points out the strength and weakness of the pathologists [7].

Continuing Medical Education All the laboratory personnel should take active participation in the various workshops, CME, seminars, etc.

27.4 Laboratory Organization

The organization of the pathology laboratory has three essential parts:

1. Laboratory construction, equipments, etc.
2. Laboratory staffs
3. Organization set-up and laboratory protocol

27.4.1 Laboratory Construction, Equipments, etc

The laboratory access pathways should be as follows: sample collection, sample processing and staining and reporting, followed by the post-examination area.

Physical aspects of the rooms:

1. *Location*: Other than the specimen and the report collection rooms, all other laboratory rooms should be inaccessible to the patients and other trespassers.
2. *Rooms*: The rooms should have the following:
   - All the laboratory rooms should be well ventilated with high ceilings.
   - The wall of the laboratory should be well painted.
   - The floor and wall should be made in such a way that they can be cleaned easily by disinfectant.
   - The room should have water supply, proper racks and closed almirah to keep the hazardous chemicals separately. The processing room must have a safety cabinet.
   - The screening room should be isolated, spacious and free from any noise.
   - The rooms for the secretarial staffs should have adequate space for the typing equipment and furniture.
3. *Safety arrangement*: The room should be equipped with proper safety arrangement such as fire extinguishers, etc.

27.4.2 Laboratory Staffs

The laboratory staffs should be in the following categories:

- Laboratory directors
- Consultant pathologist
- Biomedical scientist or technical chief
- Cytology screeners
- Laboratory technicians
- Clerical staff
- Others: Cleaner, receptionists, etc.

Job Description The duties and responsibilities of the different category of the staffs should be specified at the time of recruitment.

Qualifications and Training The technical staffs and the pathologists should have proper qualification and licence. Periodic evaluation of the staffs should be done.

27.4.3 Organization Set-Up and System Protocol

The overall organization process of the sample is important for the maintenance of good quality work. Each laboratory should have a documented plan of the scope of the laboratory service, flow chart of the work plan, allocated budget in the different areas and proper quality planning with periodic review of the whole laboratory work process.

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28 Laboratory Safety and Laboratory Waste Disposal

Each laboratory should have overall safety precautions. The laboratory safety officers should look after this following issue:

Overall security: This involves the general security of the laboratory such as safety of the equipment and reagents and prevention of entry of any unwanted persons.

A. *Security*:
   - Proper security of the laboratory staff, chemicals and valuable equipment is mandatory.
   - Entry of unauthorized person should be restricted to the laboratory.

B. *Fire hazards*:
   - The fire extinguishers, smoke alarm and fire blankets are necessary in every laboratory.
   - The laboratory personnel should know the basic operation protocol of the fire equipment.

C. *Chemical hazards*:
   - The toxic and inflammable chemicals should be in closed door fireproof metal cabinet with original labels.
   - Never do suction by mouth.
   - Always put the alkali or acid in water during the procedure of dilatation.
   - Facilities to wash eye and shower in case of toxic exposure.
   - Wear gloves, mask and laboratory coat during dealing with chemicals.
   - During preparing the diluted solution, the concentrated acid or alkali should be added in water.

D. *Infective*: The laboratory personnel should always take universal precautionary measures because we do not know the HIV status of a sample [1, 2].
   - *Universal precautions (Box 28.1)*: Health education of the technical staff regarding the universal precaution is very important to prevent the transmission of infection. Universal precautionary measures imply that all the patients should be treated as a potential source of blood-borne infections.
   - *What is it?* Universal precautions indicate to take adequate measures to prevent contact with various body fluids of the patients. Various barrier measures are taken to avoid contact with body fluids that are the potential sources of transmission of infection.
   - *The high-risk pathogens*: The pathogens that cause serious health hazards are hepatitis B, hepatitis C and HIV.
   - *Body fluids that need universal precautions*: This includes blood, peritoneal and pleural fluid, CSF, semen, vaginal secretions, synovial effusion and faecal material.
   - *Body fluids that do not need universal precautions*: These are faecal material, urine,

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nasal secretions, sweat, vomitus and sputum provided these materials are not contaminated with blood.

Universal precautions proper (Fig. 28.1):

- *Hand-washing*: Simple maintenance of hand hygiene is the single most important factor to prevent the transmission of infection. The cleaning of hands by anti-infectant soap removes the bacteria. The approved alcohol-based products such as gel or foam are superior as these substances have better microbicidal activity and do not produce drying effect. Moreover no water is needed to clean with alcohol-based cleaning agent.
- *Gloves*: Gloves prevent the blood or contaminated substances to have direct contact with the skin. Latex or nitrile gloves are better than vinyl gloves. The gloves should not be washed for reuse as the removal of the microorganisms is not always possible from the gloves.
- *Isolation gown*: The isolation gown helps to prevent contamination with blood or mucus product with the skin. It should only be worn when there is a chance of contamination of blood or blood products. For routine laboratory work, wearing of simple laboratory gown is enough.
- *Mask*: The mucus membranes of the upper respiratory tract are vulnerable to infecting agents. Mask prevents transmission of infection from the patient to the healthcare personnel or vice versa. The masks may be of variable sizes with different filtration capacities. The types of the mask depend on the need of the particular staff.
- *Goggles*: The various viral infections and *Staphylococcus aureus* can be transmitted by direct contact of splashed blood or touching the eye with the contaminated hand to the eye mucosa. Goggles should be used to prevent the transmission of infection through the eye mucosa.
- *Precautions from the sharp objects [3, 4] (Box 28.2)*: The most important pathogens that can be transmitted through needle prick injury are HIV, HBV and HCV. The injury may happen during (1) recapping the needle, (2) transfer of the blood from container to container and (3)

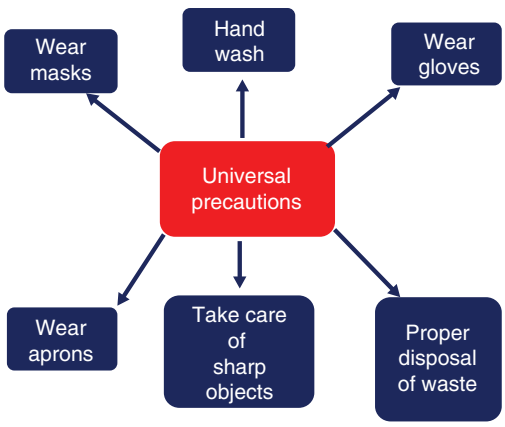

Fig. 28.1 Schematic diagram of universal precautionary measures in laboratory

Box 28.1: Universal Precautions

Universal precautions mean to take adequate measures to prevent contact with various body fluids of the patients.

- Infective agents with serious health hazards: hepatitis B, hepatitis C and HIV
- Body fluids that need universal precautions: blood, peritoneal and pleural fluid, CSF, semen, vaginal secretions, synovial effusion and faecal material
- No need for universal precautions: faecal material, urine, nasal secretions, sweat, vomitus and sputum unless contaminated with blood
- Measure:
  - Hand-wash
  - Gloves
  - Mask
  - Goggles
  - Apron
  - Take caution with sharp objects
  - Proper discarding of the contaminated waste
  - Cleaning the area

Box 28.2: Needle Stick Injury

Common causes

- Recapping the needle (25% cause)
- Inadequate disposal of needle
- Transfer of fluid from one to other container

Avoidance

- Use alternative safe technique if possible.
- Do not recap the needle.
- Use needle cutter.
- Dispose needle promptly.
- Universal precautionary measure.
- Health education.

What to do in case of needle prick

- Immediately report to your employer.
- Take appropriate care and follow-up such as post-exposure prophylaxis.
- Report to appropriate authority.

Possible transmission of diseases

- Hepatitis B (6–30%)
- Hepatitis C (1.8%)
- HIV (0.3%)

Factors that determine infection

- Immunity status of the victim: whether vaccinated or not
- Severity of the prick: superficial or deep
- Appropriate post-exposure prophylaxis
- Type of pathogen

improper disposal of the needle. Following precautions are helpful in needle stick injury:

– Do not bend or recap the needle.
– Use needle cutter to cut the needle.
– Put all the sharp objects in proper container.
– Take universal precautionary measures.
– Written document on post-exposure situation.
– Post-exposure follow-up and evaluation.

- *Sick person*: The laboratory workers with skin lesions such as weeping dermatitis or open wound in the hand should be refrained from the laboratory work area.

Standard norms in the laboratory

- Do not smoke, eat or drink within the laboratory.
- Always wear laboratory gown and gloves when you work in the laboratory.
- Do not wear sandals and shoes with open toes.
- Always avoid pipetting by mouth.
- Always clean your hand and face after removing the gloves.
- Never recap the used needle.
- Always dispose the sharp objects in the leakproof metal container.
- Always clean the laboratory area before and after working with 10% sodium hypochlorite solution.
- Keep the compressed gas cylinders under secured condition.

28.1 Laboratory Waste Disposal

All the laboratory staffs should take active participation in the proper disposable of the laboratory waste material. The different categories of the waste materials are highlighted in the table:

(a) General waste
(b) Biohazardous waste
    - Chemical waste
    - Biological waste
    - Radioactive material

Basic Norms to Dispose the Waste Material The basic norms to dispose the waste material are:

- Do not store waste in metal container.
- Do not store the chemical waste under the fume hood.
- Properly label the containers of the waste.
- Store waste only in a closed container which is leakproof and no chance of puncture in case of sharp material.

Table 28.1 Types of waste and their treatment

Colour of the bag	Waste material	Container	Disposal
Black	• Discarded medicinal substances: outdated or remnants of medicine	Plastic bag	Local authority for routine waste disposal
	• Various chemicals used for disinfection		
Yellow	• *Human anatomical waste*: human anatomical organ, body parts	Plastic bag	Disinfection and subsequently incineration
	• *Anatomical waste*: body parts of animal, various waste material of animals, discharge, etc.		
	• *Microbiology and biotechnology wastes*: wastes developed from laboratory cultures, toxin, etc.		
	• *Solid-contaminated waste*: materials contaminated with blood such as cotton, linen, bed		
Red	• *Solid-contaminated waste*: materials contaminated with blood such as cotton, linen, bed	Container with disinfectant	Disinfection and subsequently incineration
	• *Solid waste*: disposable items such as catheter		
Blue	• *Sharp materials:* blade, needle, broken glasses, etc.	Closable, puncture-resistant and leakproof hard plastic container	Incineration

- Use only one type of container for the particular type of waste.
- Do not keep incompatible chemicals in a same container such as acid and alkali should not be kept together.

Table 28.1 highlights the different types of waste and their treatment protocol.

28.2 Disinfectant Used in for the Contaminants

A. *Chlorine-Based Compounds*:
   - *Sodium hypochlorite (NaOCl)*: It is a rapid oxidant material and is a broad spectrum disinfectant. Chlorine is generated from the diluted mixture and works as disinfectant. For laboratory purpose 10% sodium hypochlorite solution is used as chemical disinfectant. The solution should be made fresh every day. As the sodium hypochlorite generates chlorine, so it is highly corrosive and should not be kept in a metallic container.

B. *Iodophors*: Iodophores are the disinfectant containing iodine in aqueous solution. Betadine and povidone-iodine are widely available commercially.

C. *Quaternary Ammonium Compounds*: These are also good detergents and disinfectants. These compounds act against various bacteria and viruses. However their action diminishes with organic matter and many detergents. These compounds are good for cleaning the laboratory floor.

D. *Phenolics*: These are derivatives of phenol and act by damaging the membrane of the bacteria and fungi. Lysol is the widely available commercial product. Many phenolic compounds are inactivated by hard water, and so it is preferable to dilute them with distilled water.

E. *Others*: Acid, alkali and alcohols are also used as disinfectants.

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