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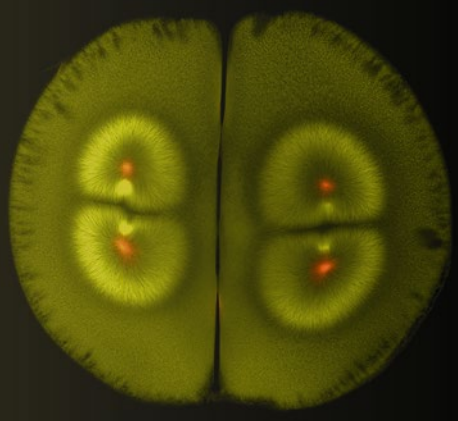

Francisco J. Pelegri *Editor*

Vertebrate Embryogenesis

Embryological, Cellular, and Genetic Methods

Second Edition

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Humana Press

METHODS IN MOLECULAR BIOLOGY

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Second Edition

Edited by

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-9008-5 ISBN 978-1-4939-9009-2 (eBook)
https://doi.org/10.1007/978-1-4939-9009-2

Library of Congress Control Number: 2018966818

This Humana Press imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

Despite continuous and fast-paced progress, much remains unknown in our understanding of biological processes. No exception is the field of animal embryogenesis, even as this field was one of the earliest to gain attention with detailed genetic, cellular, and molecular analyses. This is particularly true for vertebrate species, due to their relative intractability compared to invertebrates. Yet vertebrate systems, and their earliest stages of development, continue to both capture the attention and imagination of scientists worldwide. Due to the conservation of cellular and signaling pathways, the study of animal embryogenesis also provides valuable entry points into processes acting through the rest of organismal development and cellular homeostasis.

Within vertebrates, a small number of model systems have provided a wealth of information. These select few, including amphibians such as the African toad *Xenopus laevis*, the chick, mice, and later the zebrafish, have acted as pillars that have allowed the development of unifying hypotheses. As this general approach expands our platforms of knowledge, additional vertebrate species are being used to support or challenge those hypotheses. For example, we find that features of many model systems, such as rapid developmental time and large fecundity rates, may reflect specialized adaptations, some of which may be unique to specific phylogenetic branches. Less fecund species, which are often less convenient to use in a laboratory setting, may represent biological mechanisms and conditions that may be more widely conserved. We realize that our knowledge of animal processes is patchy, following lone branches separated from others by millions of years' worth of evolutionary changes. Cellular systems, signaling pathways, and molecules are conserved and shared, but there are innumerable differences that in our broad strokes picture appear saltatory. Given this structure of knowledge, the precise gradation of changes from one species to the other is at times hard to discern.

These gaps in knowledge are increasingly being addressed through the study of other, nontraditional vertebrate species. Nontraditional vertebrate model systems have also recently gathered tremendous momentum through the development of readily implemented gene editing tools, such as CRISPR-Cas9. This new technology enables targeted changes to the genome and is especially useful for generating loss of function conditions to assess gene function. The limits of our knowledge are no longer set by high fecundity and short generation time, and any organism that can be bred under controlled conditions is now accessible for the genetic analysis of endogenous functions and mechanisms.

This volume has been compiled within this context and accordingly represents a continuum between the traditional model organisms and new methodologies and model systems. Improved protocols with updated advances in key traditional model systems are included, such as in amphibians, chicken, mouse, and zebrafish. Within these traditional model systems, new developments are presented, such as protocols for the analysis of cellular membranes and intracellular signals, light-controlled manipulation of function, and the analysis of transcriptomic and proteomic data in the context of the embryo. Additional systems are described, including the leopard gecko and the flexible-shelled slider turtle.

Several other timely topics relevant to vertebrate embryogenesis are presented in this volume. Recent studies have highlighted the importance of membrane-less compartments as cellular functional hubs in the embryo. In this volume, a series of chapters addresses the

visualization and characterization of the balbiani body, a membrane-less structure involved in the oocyte and primordial germ cell development. An additional set of chapters addresses reproductive technologies for gene function assessment and germ line manipulation, such as interspecies hybrid production, germ line transplantation, and somatic cell nuclear transfer. Another chapter provides a simple protocol for the induction of gene-inactivating CRISPR-Cas9-mediated mutations. As a presage to a future with an increasing contribution of mathematical analysis to developmental and cellular biology, a chapter includes detailed directions to model cell division patterns in early embryos. Finally, this volume includes an updated chapter on the responsible care and use of vertebrate animals in a laboratory setting.

As with the first edition of this series, this volume is meant to foster cross-model work and ideas by presenting methods that can be applied across laboratories and species boundaries. We are also delighted to present chapters on new model systems. We hope that this work will be useful to scientists, educators, and the advanced general audience and will act as an inspiration to further an understanding and appreciation of animal development.

Madison, WI ***Francisco J. Pelegri***

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Chapter 1

Culture and Host Transfer of *Xenopus* Oocytes for Maternal mRNA Depletion and Genome Editing Experiments

Douglas W. Houston

Abstract

The early development of *Xenopus* critically depends on maternal components stored in the egg. Because important events such as axis formation are triggered immediately after fertilization, it is often desirable to perturb gene function before this occurs. Oocytes can be experimentally manipulated in vitro, prior to maturation, and subsequently fertilized or otherwise activated to develop, and then observed for any embryological defects. Available methods for fertilizing cultured oocytes include in vitro fertilization following oocyte vitelline envelope removal, nuclear transplantation, intracytoplasmic sperm injection, and transferring oocytes to the body cavity of ovulating host females (host transfer). This chapter outlines this host transfer method, which has been used to elucidate basic mechanisms of axis formation, germ-layer induction, and primordial germ cell specification. Methods for obtaining, culturing, transferring, and fertilizing *Xenopus* oocytes are described. This method has typically been used to alter maternal gene function by antisense oligonucleotide-mediated mRNA knockdown, but is also useful for mRNA or protein overexpression, including the expression of genome-editing reagents prior to fertilization.

Key words *Xenopus*, Maternal genes, Antisense, Host-transfer, Oocytes

1 Introduction

Important events in early *Xenopus* development are regulated by maternal proteins and mRNAs that are synthesized and stored during oogenesis, and in some cases differentially localized in the oocyte to regionally direct subsequent cell fates in the embryo (reviewed in Refs. [1, 2]). For rapidly developing invertebrates and small vertebrates (*Drosophila*: [3]; *C. elegans:* [4]; zebrafish: [5, 6]), maternal gene function can be assessed by generating maternal-effect mutations. However, long generation times and allopolyploidy have historically rendered this approach impractical for *Xenopus spp.* and other amphibians. However, with available genome sequences [7, 8] and genome editing methods (reviewed in Ref. [9]), generating such "reverse-genetic" maternal-effect mutations in *Xenopus* is now within the realm of feasibility.

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_1,

Producing these mutants still requires significant time and would not be suitable in cases where mutations affected overall survival or germline development. Thus, there will likely be a continued need for methods to rapidly assess the function of genes with a significant maternal contribution.

Xenopus spp. have several distinct advantages for studying maternal gene function during development. Substantial new zygotic transcription of mRNAs does not begin until the 4000-cell mid-blastula stage (the mid-blastula transition, MBT) in *Xenopus* [10–12], and important events in early development occur during this pre-MBT period. These include the establishment of the dorsoventral axis by cortical rotation, formation of the primary germ layers, and specification of the primordial germ cells (reviewed in Refs. [1, 2]). Additionally, the oocytes, eggs, and early embryos are all robust to manipulation, contain abundant mRNA and protein for biochemical characterization, and have been extensively fate mapped so that the contribution of different regions of the egg and embryo to embryonic tissues is known with a good deal of precision.

Most gene function studies in *Xenopus* have focused on either overexpression studies or functional inhibition, injecting dominant-negative constructs or antisense oligonucleotides (oligos), either of the DNA or the morpholino variety, into the fertilized egg (reviewed in Ref. [13]). While these methods are clearly effective in many cases, and are convenient to perform, it is often not possible to assess early maternally controlled processes by injecting antisense reagents into fertilized eggs/embryos. There is approximately a 2-h time lag between the injection of an mRNA and significant translation of the encoded protein, and morpholino oligos do not instantly inhibit target mRNA translation, requiring the protein target to be turned over, often at a slow or unknown rate [14]. Also, events such as cortical rotation and aggregation of the germ plasm occur just after fertilization, thus allowing only a short time window available for interference.

The use of RNase H-dependent DNA oligos was initially investigated in *Xenopus* oocytes [15–17], which are tolerant to injected oligos and do not rapidly degrade oligos with phosphorothioate modifications [18, 19]. By contrast, most oligo types are generally highly toxic and unstable when injected into the egg milieu [19]. Newer generations of oligo modifications reducing toxicity and increasing affinity and stability have been developed [20] but these are not usually commercially available and have unfortunately not been widely adopted. Thus, oocytes are advantageous for antisense experiments, but cannot be directly fertilized, owing to a lack of surface modifications and jelly coats normally acquired during transit through the oviduct [21]. Additional steps must be taken to prepare these oocytes for fertilization in order to assess the developmental outcomes of any oocyte manipulations. The most widely

implemented of these methods is a classic method of transferring mature oocytes into the body cavity of an ovulating host female (i.e., host transfer; Ref. [21, 22]). This method was revived by Heasman and Wylie (*see* Ref. [23]) and used to study the effects of ultraviolet irradiation and antisense depletion of *keratin 8* mRNA on the subsequent development of treated oocytes [24, 25].

Subsequently, the host transfer method has been used in seminal experiments identifying maternal requirements for beta-catenin in dorsal axis formation [26], localized *vegt* mRNA in germ-layer pattering [27] and germ plasm-localized *dazl* mRNA in germline specification [28] among others. Underscoring the importance of studying maternal contributions in oocytes, unique effects on microtubule organization can be observed following depletion of *tripartite motif containing 36 (trim36)* or *dead end homolog 1 (dnd1)* in oocytes [29, 30], whereas other defects are seen when the same reagents are injected after fertilization [31, 32]. Similarly, depletion of *tcf7l1/tcf3* or *vegt* mRNAs in oocytes results in different effects than does their inhibition after fertilization [27, 33–35]. Thus, the elimination or inhibition of maternal gene products in the oocyte prior to fertilization is often necessary to identify the earliest function of those genes in early development.

The host transfer method was first used in the early part of last century and relies on the normal transport mechanisms for ovulated amphibian eggs: the movement of eggs in the body cavity into the ostium and through the oviducts, driven by peritoneal cilia [21]. During this passage, the eggs acquire their mucus jelly coats, which are needed for sperm attraction, activation, and subsequent egg fertilization. This transport was first shown using buckshot and body cavity (coelomic) eggs from a donor *Rana* female transplanted into a host female's body cavity [21]. Later, the transfer of body cavity eggs between several amphibian species was used to study the role of jelly coats in overall fertilization and in species-specific sperm recognition (*Triturus:* [36]; *Hyla, Bufo, Rana*: [37]; *Rana:* [22, 38, 39]). These studies were extended by showing that ovarian oocytes (still in diplotene of prophase I) could be matured in vitro (to metaphase II eggs) following hormone (progesterone) treatment and still undergo normal fertilization and development following transfer to host females (*Rana*: [40]; *Xenopus*: [41]).

Other methods to fertilize jelly-less eggs or matured oocytes have been tried with varying degrees of success, including proteolytic treatment or removal of the vitelline membrane along with incubation of sperm by jelly-conditioned water (*Rana*: [42]; *Bufo:* [43]; *Xenopus*: [23, 44]), nuclear transplantation (*Rana:* [45–47]; *Xenopus*: [48]), and intracytoplasmic sperm injection (*Xenopus:* [49–53]).

This protocol details the oocyte host transfer for isolating and fertilizing cultured oocytes in *Xenopus*. Although this procedure is lengthy, and some techniques require a degree of practice, often causing some trepidation regarding its use, there are many distinct advantages to the host transfer method compared to other methods of fertilizing cultured oocytes. First, little specialized equipment is needed beyond a few surgical tools, and complex treatment of sperm or oocytes is not required. Second, the method may allow for more physiologically relevant outcomes since there are no enzymatic treatments of oocytes involved. Consequently, success rates are higher for host transfer, with up to 50% or more of oocytes being recovered and developing normally. Last, the adoption of intraperitoneal injection has greatly simplified the oocyte transfer and lessened the regulatory burden, since this procedure is not classified as a survival surgery. If oocytes are obtained commercially or by sacrificing the female, this method can be performed without including surgery in the animal protocol, a significant barrier for many labs. Recent improvements to the method include transplantation by intraperitoneal injection and modified culture medium. These methods would be suitable for the overexpression of mRNAs, including tagged constructs, as well as for using antisense oligos to deplete mRNAs. The details regarding oligo design and use are described elsewhere [54, 55]. Recently, pre-fertilization injection of genome-editing reagents has been used for highly efficient mutagenesis and homology-directed repair in the F0 generation in both *Xenopus laevis* and *X. tropicalis* [53, 56–58], prompting another revitalization of interest in performing these methods. With genome editing and related methods are rapidly becoming de rigueur in developmental biology, it is likely that the ease and robustness with which oocytes can be manipulated in vitro and fertilized will greatly facilitate genetic studies in *Xenopus* and potentially other amphibians where similar methods are available.

I note that this protocol is an updated and streamlined version of that in Olson et al. [55].

2 Materials

All solutions are made using deionized, ultrapure water and are prepared in clean glass- or plasticware. Reagents should be relatively new (<1 year old), of high quality, and obtained from reliable sources.

2.1 Surgical Isolation of Donor Ovary Tissue

1. Anesthetic solution: MS222 (ethyl 3-aminobenzoate methanesulfonate salt): 1.0 g/L MS222, 0.7 g/L sodium bicarbonate, final pH ~7.0.

2. Modified oocyte culture media (OCM): 67% Leibovitz's L-15 (Gibco™, + L-glutamine, 2 mM), 0.05% polyvinyl alcohol (PVA), 1× Pen-Strep: adjust pH to 7.6–7.8 with NaOH, make fresh, and store at 14–18 °C for up to 1 week.
3. Surgical and dissection instruments (Fine Science Tools): Scalpel handle (#3) and blade (#10 or 11), several pairs of Dumont forceps (#4 or #5 Biologie), Bonn iris scissors (curved or straight), Halsey or Olsen-Hegar microneedle holders.
4. Clear sterilization pouches with steam indicator strip (for sterilizing surgical instruments).
5. Surgical sutures: 4-0 PDS II Violet monofilament with 17 mm ½ circle needle, RB-1 taper (Ethicon). 5-0 PDS II sutures are used for *X. tropicalis* surgeries.
6. Sterile surgical gloves.
7. Frog water: Tap water treated with Amquel (chloramine remover).
8. Petri dishes: Large (100 mm)- and medium (60 mm)-sized plain sterile Petri dishes.

2.2 Isolation, Culture, and Microinjection of Oocytes

1. Several pairs of Dumont forceps (#4 or #5 Biologie), dedicated to oocyte or embryo work (not used for surgeries).
2. Petri dishes: Large (100 mm)- and medium (60 mm)-sized plain sterile Petri dishes.
3. Microinjection needles, e.g., Drummond replacement capillaries, pulled to desired specifications.

2.3 Preparation of Donor Oocytes and Host Females for Transfer

1. Progesterone (4-Pregnene-3,20-dione, Sigma): 10 mM Stock in 100% ethanol (used as 5000× stock), store at −20 °C.
2. Human chorionic gonadotropin (hCG): 10,000 IU/vial, reconstitute with 5 mL sterile water (2000 IU/mL), store at 4 °C for up to 30 days.
3. Vital dyes: Stock solutions of the "Blue," "Red," and "Brown" dyes are made in 50 mL deionized water, incubated for 20 min with rocking, and spun in a clinical centrifuge. Aliquots (~1 mL) are taken from the supernatants and stored at −20 °C:
   (a) 0.1% Nile Blue A: 0.05 g/50 mL
   (b) 0.25% Neutral Red: 0.125 g/50 mL
   (c) 1% Bismarck Brown: 1.0 g/50 mL.

2.4 Oocyte Transfer by Intraperitoneal Injection

1. Anesthetic and OCM (see above).
2. 2 mL Glass syringe (Tomopal Inc.), with a luer lock adaptor.
3. 16 gauge, 1 in. (16G1), sterile syringe needles: 19- or 20-gauge needles are used for *X. tropicalis* oocyte transplantations.

2.5 In Vitro Fertilization of Host-Transferred Oocytes

1. 10× Marc's Modified Ringer's (MMR)–triple HEPES: 1 M NaCl, 18 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 150 mM Hepes, adjust to pH 7.8 with 5 N NaOH, filter sterilize and store at 4 ° C. Adjust pH to 7.6–7.8 when making 1× or 1.2× (high-salt) solutions; make dilutions of 0.3×, 0.1×, and 0.05× from the 1× stock.
2. 2% Cysteine in 0.1× MMR, pH to ~7.8, check with pH paper. 3% Cysteine in 0.1× MMR is used for dejellying *X. tropicalis* embryos.
3. Teflon pestles for 1.5 mL microfuge tubes (Kimble-Kontes).

3 Methods

All procedures are done at room temperature unless otherwise specified. Follow all relevant guidelines set by the Institutional Animal Care and Use Committee (IACUC) or equivalent compliance body. *See* Fig. 1 for a diagrammatic overview and a general

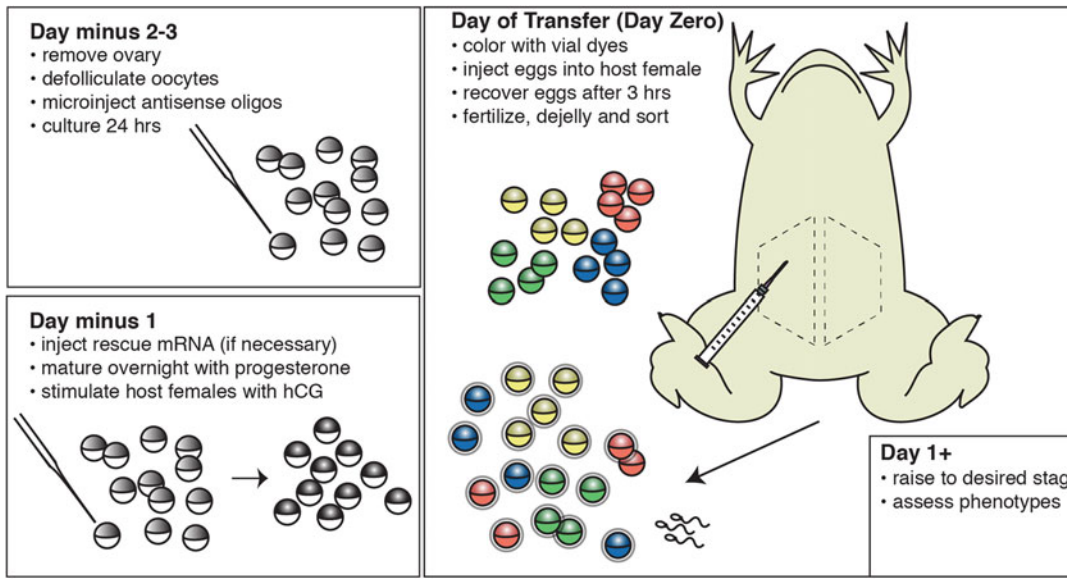

Fig. 1 Outline of the host transfer procedure by intraperitoneal injection. On day minus 2–3, ovary is removed and oocytes are defolliculated. Oocytes are then injected with mRNAs or with antisense oligonucleotides targeting the endogenous mRNA of the candidate gene. On the next day (day minus 1), rescue mRNA is injected (if necessary), the oocytes are matured with progesterone, and prospective host females are induced with hCG. On the day of the transfer (day zero), the oocytes are colored with vital dyes, and injected into an egg-laying host female. Once the eggs are released, they are fertilized, sorted from host eggs (i.e., noncolored eggs, not shown), and analyzed as desired. The dotted areas indicate optimal regions in the abdomen for inserting the experimental oocytes

timeline of the procedure. These methods are most widely used for *X. laevis,* but modifications for transferring *X. tropicalis* oocytes into either *X. tropicalis* or *X. laevis* host females are described where appropriate.

3.1 Surgical Isolation of Donor Ovary Tissue

1. Ovary is surgically dissected from anesthetized females. Survival surgery is preferred so that females with high-quality oocytes may be used multiple times. Survival surgeries are done using aseptic technique (*see* **Note 1**).
2. Prepare fresh oocyte culture medium (OCM) media prior to performing surgery and pour into dishes ahead of time as desired.
3. Prepare the surgical area and sterilize the instruments. Disinfect a dedicated surgical area using 70% EtOH. Open a pouch of sterile instruments and arrange them as needed on the sterile inside of the package. Open a sterile suture package, clamp the needle into the microneedle holder and set on the sterile inside surface.
4. Select several females dedicated for ovary removal and place one in anesthetic solution. Check periodically that a surgical plane of anesthesia is achieved by turning the frog onto the back and by pinching a toe. Anesthetized animals will be unresponsive. If animals are not completely anesthetized, place back into anesthetic solution and check at 5-min intervals until anesthesia is complete.
5. Place an anesthetized female in dorsal recumbent position on a damp wipe and cover the head and legs. Create a sterile field over the frog by placing a sterile wipe over the animal and wetting with sterile water. Tear a small hole in the wipe over the abdomen to form a surgical window.
6. Make a 1 cm incision in the skin in the lower part of the abdomen using small iris scissors or scalpel. Use forceps to grasp the underlying muscle, pull upward, and incise sharply with a single clean cut to expose the body cavity. The ovary should be immediately visible or can be carefully probed for using blunt forceps.
7. The ovary has about 24 lobes, each containing hundreds of oocyte follicles [59]. Pull several of the lobes through the incision and trim away exposed tissue. Place the ovary piece into a large (100 mm) Petri dish of OCM. Repeat until the desired amount is obtained. Six to ten large lobes are sufficient for a typical experiment (*see* **Note 2**).
8. Close the muscle and fascia layer first with simple interrupted suturing, using basic instrument ties to place surgeon's square/reef knots several millimeters apart. Once the body wall layer is secure, proceed to close the skin in a similar manner (*see* **Note 3**).

9. Rinse the frog in water and place in a shallow recovery bucket. The container is inclined so the water does not cover the frog's nostrils. Cover the container and allow the animal to recover before moving it to a larger recovery bucket. Recovery is indicated by response to touch, eye bulging, and eventually purposeful movements. Return to the colony after the frog is fully recovered and continue monitoring for several days.
10. Subdivide the extracted ovary into smaller pieces using sterile iris scissors (re-sterilized in a bead sterilizer if available). Cut open one side of an individual lobe and flatten out the tissue. Continue cutting into small pieces, about 2 cm^2, and the pieces are transferred to a dish of clean OCM, keeping about six pieces per dish. Dividing the ovary in this way extends the life of the tissue in culture and makes defollicating a bit easier (*see* below). Culture for up to 4–5 days in OCM at 18 °C, or up to 2 days at 25 °C for *X. tropicalis* ovary.

3.2 Isolation, Culture, and Microinjection of Oocytes

1. Oocytes for host transfer must be manually defolliculated because collagenased oocytes are not fertilizable. Defolliculate healthy, fully grown stage VI oocytes, identifiable by larger size (1.2–1.3 mm diameter (*X. laevis*) or ~0.4 mm (*X. tropicalis*)) uniform animal pole and, in some cases, a distinct equatorial band of lighter pigmentation (*see* **Note 4**).
2. For manual defolliculation, using the nondominant hand, grip the connective tissue theca layer near a desired oocyte with a pair of watchmaker's forceps. With a second pair of forceps, lightly grasp the tissue adjacent to the oocyte and tear into the theca layer and continue pulling away from the ovary. The oocyte can be teased out through the resulting slit in the connective tissue layers.
3. Successfully defolliculated oocytes are flaccid and do not have visible blood vessels (these are part of the thecal layer). Oocytes retaining follicle will not be easily injected with typical microinjection needles. Experienced operators can easily isolate 200–400 oocytes per hour. Transfer groups of defolliculated oocytes to medium (60 mm) Petri dishes using a sterile glass Pasteur pipette. Culture groups of 75–200 oocytes at 18 °C (or 25 °C for *X. tropicalis*) (*see* **Note 5**).
4. Microinject oocytes with antisense oligos or RNAs as desired. The oocytes can be injected directly while in OCM. Typical injection volumes are 5–10 nL, delivering doses of 2–6 ng of DNA-based oligos, 5–100 ng of morpholino oligo, 20 pg to 1 ng of mRNA, or 1–2 ng of purified protein (e.g., Cas9 proteins). For *X. tropicalis*, ~3 nL is injected per oocyte, with corresponding reduction in reagent dosage.
5. Injected oocytes are transferred to 60 mm dishes containing 8 mL of fresh OCM and cultured as above. Because of limitations

on the number of colors that can be made using vital dyes (see below), only 5–6 groups (~75–200 oocytes each) can be transferred per female, so experiments must be planned with this in mind. Oocytes injected with DNA-based oligos should be cultured for at least 24 h to allow for degradation of the oligo and target mRNA before proceeding with the host transfer. Culture can be extended to 72 h without affecting oocyte viability or developmental capacity of the resulting embryos.

3.3 Preparation of Donor Oocytes and Host Females for Transfer

1. On the night before the transfer, usually day one after defolliculation and injection, oocytes are stimulated to undergo maturation by addition of progesterone to a final concentration of 2 μM (1.6 μL of 10 mM stock/8 mL OCM). The dishes are swirled to mix and returned to 18 °C overnight. For *X. tropicalis* oocytes, treat with progesterone the morning of the transfer (these "trop" oocytes can be transferred either into *X. tropicalis* host females, *see* **step 7** below, or into *X. laevis* hosts and fertilized with *X. tropicalis* sperm; *see* **Note 6**).
2. Around the same time, inject several females (2–5 animals) with hCG (1000 U) to induce ovulation and leave at room temperature or 18 °C overnight. Host transfer experiments work best in our hands if progesterone treatment of oocytes and hCG injection of females are done around the same general time, within ~30 min of each other (for *X. laevis*, both treatments can be done about 10–12 h prior to oocyte implantation). Multiple females are injected to ensure that at least one will have suitable egg quality to serve as the host (*see* **step 5** below).
3. The next morning, check that the oocytes have matured (i.e., undergone germinal vesicle breakdown (GVBD), evidenced by white spot formation at the animal pole; *see* **Note 7**) and that the females have begun spawning. If any oocytes became stuck together overnight, gently tease them apart to avoid clogging during intraperitoneal injection. A sample of oocytes (usually 3–5 oocytes) should be frozen on dry ice at this point for verification of mRNA or protein knockdown or expression.
4. Thaw vital dye stocks and spin briefly to remove particulates. Add ~80 μL of stock to each 60 mm dish containing the oocytes, followed by gentle swirling. Combine multiple appropriate dyes if certain colors are desired. Incubate with rocking for 10–15 min, until the desired color level is reached. Transfer colored eggs to a large dish of OCM to wash. Hold until implantation (*see* **Note 8**).
5. While the oocytes are staining, choose a host female and place in anesthetic solution. Choose animals that have just started laying healthy eggs and can be induced to release more eggs upon gentle squeezing. Healthy eggs will be firm and uniformly pigmented in the animal pole and generally have a distinct pale band around the equator. Avoid females that are

laying string eggs or that crush eggs upon manual extrusion from the oviduct, both of which indicate poor reproductive quality of the host.

6. Place the anesthetized female in dorsal recumbent position on a damp wipe. Cover the head and legs as above and gently blot away anesthetic liquid with the edge of the wet wipe.
7. For homotypic transfers of *X. tropicalis* oocytes into *X. tropicalis* females, both females and a male are primed overnight with 10 U hCG and boosted the next morning with 200 U hCG, with animals being kept at 25 °C throughout. Similarly, *X. tropicalis* oocytes are stimulated with progesterone in the morning at the same time as the boost injection. *X. tropicalis* females are anesthetized for transfer about 2 h after boosting, usually before egg-laying begins (usually after 4 h). For transfers into *X. tropicalis,* it is essential to transfer about 2 h prior to egg-laying to maximize the recovery of transferred oocytes.

3.4 Oocyte Transfer by Intraperitoneal Injection

1. Fit a 16 gauge, 1 in. sterile disposable needle (16G1) onto a 2 mL glass syringe. Remove the plunger and set aside (it will not be used for injection). Rinse the inside of the syringe with OCM to coat the surfaces with PVA (in the OCM), reducing sticking of oocytes. 19–20 gauge needles are used for *X. tropicalis* oocytes.
2. Angle the syringe at 45° with the beveled edge of the needle up. Insert the tip into the lower abdomen of the host female, orienting the needle toward the anterior and passing through both skin and muscle. Brace the injection site with a gloved finger to provide some support, allowing the needle to insert smoothly. The needle will have passed through both layers when resistance against the needle can no longer be felt. Do not penetrate too deeply into the body cavity (*see* **Note 9**).
3. Keeping the needle at 45° with one hand, slowly add colored oocytes along the inner wall of the syringe using a Pasteur pipette. Allow the oocytes to drain to the bottom and collect in a small volume of OCM (~1 mL).
4. After the oocytes have been added, tilt the syringe to 90° while keeping the needle in the frog. Tap gently on the side of the syringe and the oocytes will begin draining into the body cavity. Adjust the needle depth up and down if oocytes do not flow right away. Flush with OCM if some oocytes become stuck around the edge of the syringe port. Excess OCM, up to 2 mL or so, does not seem to be detrimental.
5. Drop the syringe back to a 45° angle and withdraw the needle. Oocytes should not spill out of the insertion site, although some OCM may drain out. Very little bleeding should occur.

Wash the female briefly with distilled water and allow to recover in normal frog water as above. Suturing or other means of closing the wound are not necessary.

3.5 In Vitro Fertilization of Host-Transferred Oocytes

1. After the host has recovered from anesthesia, place in a container of 1 L high-salt MMR (1.2× MMR, pH 7.6). A 4 L Nalgene plastic beaker is a convenient vessel for this purpose. Eggs released into high salt will remain competent for fertilization for many hours. For *X. tropicalis* transfers (homo- or heterotopic), 1.0× MMR with 0.05% BSA can be used.
2. Continue to monitor the host for the appearance of ovulated donor eggs. Egg-laying should continue after the procedure and colored eggs will appear 2–3 h after implantation. The female can be gently squeezed if colored eggs do not appear by 3–4 h. Rarely, the host will stop laying eggs, resulting in a failed experiment, as it is nearly impossible to recover the transferred eggs from the oviducts.
3. In the meantime (or beforehand) obtain testes from a male frog through non-survival surgery (*see* **Note 10**). Keep testes in OCM at room temperature throughout the day, and at 4 °C for longer term storage.
4. Once colored eggs appear in the bucket, the female can be "squeezed" if desired, by holding the frog firmly and applying gentle pressure to the belly using a thumb. Eggs are squeezed into a dry Petri dish and the female is returned to the high-salt MMR. Eggs are fertilized with a sperm suspension, made by homogenizing ~1/4 testis in a microfuge tube containing 500 μL 0.3× MMR, using a Teflon pestle. This suspension is added to the eggs, followed by ~3 mL additional 0.3× MMR, and fertilization is continued for a total of 4 min. The eggs are then flooded and rinsed with 0.1× MMR and left to develop. It is often convenient to dejelly and sort cleaving colored eggs at the four-cell stage (*see* **Note 11**).
5. After the female ceases egg-laying (or after about 5 h), the eggs in high salt can be fertilized. Gently squeeze out any remaining eggs and carefully drain most of the high-salt buffer. Rinse the eggs once in 0.3× MMR and carefully drain again. The eggs are transferred to a Petri dish and as much of the remaining buffer as possible is removed with a transfer pipette.
6. Fertilize the eggs as above by homogenizing half an *X. laevis* testis (whole testis for *X. tropicalis*) in a microfuge tube using a Teflon pestle, in a volume of 500 μL 0.3× MMR. Add the homogenate to the eggs and swirl to mix thoroughly and add ~3 mL 0.3× MMR. Eggs recovered from high salt tend to activate more slowly, so let them incubate for ~10 min, or until donor eggs are activated, flood and rinse with 0.1×

MMR. Colored eggs are dejellied and sorted as above and left to develop to the desired stages for analysis. For *X. tropicalis* oocytes, remember to use *X. tropicalis* testis.

4 Notes

1. Typical policies for *Xenopus* survival surgery allow for up to five surgeries, followed by a sixth terminal procedure. Regulations may differ across different institutions so be sure to check surgical guidelines established by your local IACUC or equivalent compliance body.
2. Many females will likely have ovary of sufficient quality, indicated by numerous large oocytes with uniform animal pigmentation. Not all healthy oocytes will look the same, but unsuitable ovary can usually be distinguished by the presence of dying/atretic oocytes, yellow or speckled oocytes, or a paucity of fully grown stage VI oocytes. It is generally good practice to test several females and choose the best among them.
3. The suturing technique is documented in Schneider et al. [60], and numerous Internet-based videos are also available demonstrating proper suturing. Additionally, institutional animal care offices should provide instruction in surgical methods if requested. Ovary may be obtained commercially if performing survival surgery is problematic or undesirable.
4. Manual defolliculation of oocytes requires a significant learning curve as well as considerable "hands-on" time to isolate the required numbers. However, success greatly increases with practice, and many students can easily obtain 200–400 quality oocytes per hour. Detailed descriptions of the technique can be found in Refs. [55, 60, 61]. I have also posted a video on YouTube [62]. Important considerations for defolliculating are the use of forceps that meet exactly at the tips and the use of very light pressure when closing the forceps. Any pressure beyond what is needed to make the tips just meet will increase the chances of pulling the oocyte from the ovary without removing follicle layers. Remembering to breathe, exhaling while tearing the follicle, is also helpful in achieving high success rates.
5. Since the eventual maturation response shows batch-to-batch variation, it is good practice to test maturation prior to microinjection. Early in the day, treat 20 or so oocytes with 2 μM progesterone and assess the number that undergo germinal vesicle breakdown (GVBD), about 6 h later. If more than half the oocytes fail to mature, it may be best to start again with new ovary.

6. Fertilization in this cross-species transfer is possible because *X. tropicalis* sperm are able to recognize and penetrate *X. laevis* jelly coats (but not vice versa; [63]). Some *X. laevis* host eggs will fertilize and may develop as interspecific hybrids.
7. White spot formation is commonly used as a reliable indicator of maturation. It results from the breakdown of the nuclear envelope during entry into meiosis followed by emission of the first polar body at the animal pole. The area of animal pole pigment is disrupted where the polar body is extruded, leaving a conspicuous white spot.
8. The final vital dye concentrations are as follows: Blue = 0.001% Nile Blue A, Red = 0.0025% Neutral Red, and Brown = 0.01% Bismarck Brown. Five main colors are possible, each of the single colors plus Mauve (80 μL Blue + 80 μL Red) and Green (80 μL Blue + 80 μL Brown). A sixth color can also be made, Orange (80 μL Brown + 80 μL Red), but this can be very difficult to distinguish from either Brown or Red alone and is only used as a last resort.
9. Intraperitoneal (IP) injection is preferred over surgical transplantation because it is less invasive and less potentially detrimental to the animals. Methods other than syringe needles can be used for introducing oocytes, including catheter syringes or sharpened Pasteur pipettes. Alternatively, normal abdominal surgery can be performed as previously described [55, 60]. Use caution when performing the injection to avoid self-injury by puncturing fingers or hands.
10. Testes are typically isolated from terminally anesthetized males, as described in Ref. [64]. Briefly, males are euthanized with a high dose of MS222 (10 g/L), the abdomen is incised, and the cavity is exposed. The testes are white in appearance and surrounded by blood vessels and are found at the anterior base of the yellowish fat bodies. The fat bodies are teased to the side and testes are carefully cut away from the peritoneal connective tissue.
11. The vital dye colors are most easily distinguishable when viewed from the animal pole at the 2–8-cell stage. Afterwards it is necessary to roll the embryos over to see the vegetal pole. This can be laborious if the colors are faint or if there are numerous embryos.

Acknowledgments

This work was supported by NIH grant GM083999 (DWH) and the University of Iowa (DWH).

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Chapter 2

Immunofluorescence of Microtubule Assemblies in Amphibian Oocytes and Early Embryos

Thao Nguyen, Timothy J. Mitchison, and Martin Wühr

Abstract

Amphibian oocytes and embryos are classical models to study cellular and developmental processes. For these studies, it is often advantageous to visualize protein organization. However, the large size and yolk distribution make imaging of deep structures in amphibian zygotes challenging. Here we describe in detail immunofluorescence (IF) protocols for imaging microtubule assemblies in early amphibian development. We developed these protocols to elucidate how the cell division machinery adapts to drastic changes in embryonic cell sizes. We describe how to image mitotic spindles, microtubule asters, chromosomes, and nuclei in whole-mount embryos, even when they are hundreds of micrometers removed from the embryo's surface. Though the described methods were optimized for microtubule assemblies, they have also proven useful for the visualization of other proteins.

Key words *Xenopus*, Amphibians, Immunofluorescence, Yolk clearing, Development, Embryos, Mitotic spindle, Microtubule asters, Large cells

1 Introduction

Amphibian oocytes and embryos are classical model systems to study cellular organization and vertebrate development. Swammerdam was probably the first person to describe cell division when he observed freshly laid frog eggs: "Next I observed the whole of the little frog divided, as it were, into two parts by an obvious fold or furrow" [1, 2]. A unique advantage of amphibian zygotes is their large size, which is on the order of 1 mm in diameter. This enables easy observation and manipulation. Hertwig and Pflueger deformed early frog embryos with glass plates and observed the reorientation of the cleavage plane leading to the famous Hertwig rule: the mitotic spindle cleaves the cell perpendicular to its longest axis [3, 4]. Currently *Xenopus laevis* is the predominant amphibian

Electronic Supplementary Material: The online version of this chapter (https://doi.org/10.1007/978-1-4939-9009-2_2) contains supplementary material, which is available to authorized users.

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_2,

model system for research. This is due to its year-round availability, easy in vitro fertilization, large embryos, rapid development, and ability to adapt to a wide range of laboratory conditions [5].

Besides their classical importance in studying vertebrate development, the introduction of *Xenopus* egg extract in the 1980s resulted in an important biochemical tool to study basic cell biology questions in a test tube [6–8]. *Xenopus* egg extract is essentially undiluted cytoplasm, which is considered "alive" by many accounts. Unlike more dilute forms of lysate, e.g., from tissue culture cells, the *Xenopus* egg extract can recapitulate many cellular processes in vitro. Among them are the formation of spindles, separation of sister chromatids, formation of nuclei, and recapitulation of cytokinesis as well as multiple rounds of cell cycles [9–12]. *Xenopus* egg extract has been a critical system to study spindle composition and formation, and has been more recently adapted to recapitulate millimeter-sized aster formation and aspects of cytokinesis in vitro [13–16]. More recently, *Xenopus* eggs, embryos, and lysates have become popular for quantitative proteomics experiments [17–20]. These systems are particularly attractive due to the large amount of material they provide at well-defined cellular or developmental stages. For example a single *Xenopus laevis* embryo contains ~30 μg of non-yolk protein [21].

Despite their utility in cell biological and developmental studies, amphibian eggs and embryos are difficult to image. This is due to their large size and widely dispersed yolk, rendering the embryos opaque. Nevertheless, visualizing oocytes and embryos is crucial for the interpretation of in vitro work and to connect molecular findings with underlying morphological changes. In the late nineteenth century Oskar Schultze performed pioneering work to image these structures deep inside the amphibian eggs and embryos. He visualized the meiotic spindle via thin-sectioning and some precursors of immunofluorescence (IF) [22] (Fig. 1a). However, whole-embryo imaging is hindered by yolk, which is dispersed throughout. Yolk contains crystalline proteins with higher density than cytoplasm. The light diffraction at the yolk-cytoplasm interface results in opaqueness of the embryo. Even with laser scanning microscopy live imaging is only possible close to the surface [23–25]. Kirschner and Murray proposed to replace the water in the embryos with a solution that matches the yolk's refractive index (~1.56 vs. ~1.33 of water). This solution is called "Murray's clear," consisting of a 2:1 mixture of benzyl benzoate and benzyl alcohol [26]. Murray's clear renders opaque eggs nearly transparent. Making use of this method, David Gard's lab pioneered the usage of laser scanning microscopy and IF in *Xenopus* embryos [27]. Here, we describe our adaptations of his lab's protocols for the imaging of microtubule structures in early development. We adapted these protocols to study how the cell machinery can find center and longest axes in cells that change their size within 5 h from a 1 mm single-cell

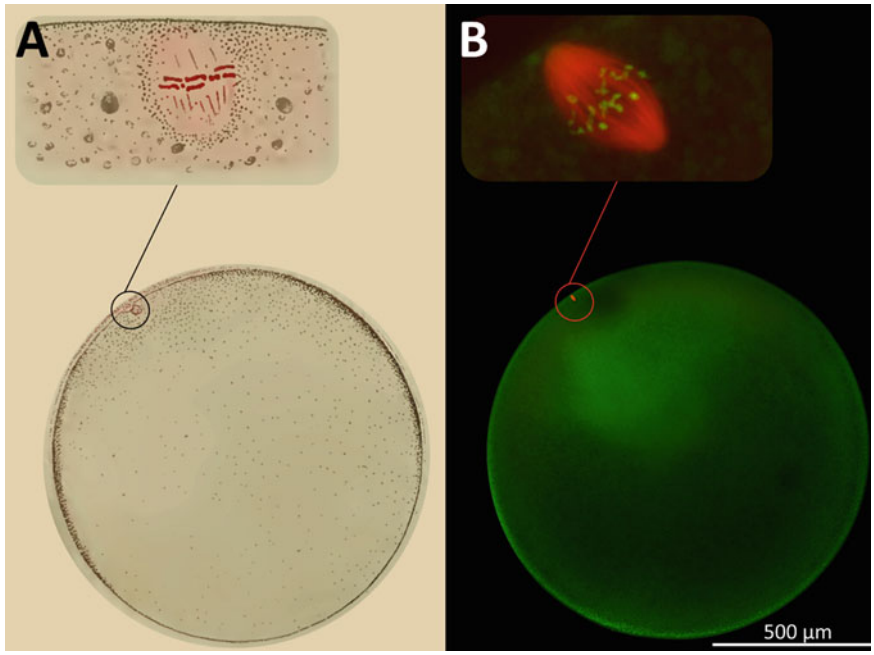

Fig. 1 Comparison of micrographs of the meiotic spindles in amphibians imaged in the nineteenth and twenty-first centuries. (**a**) Drawing of Schultze from 1887 based on observations of a meiotic spindle in the Axolotl egg obtained by thin sectioning and precursor to IF [22]. (**b**) Whole mount of a *Xenopus laevis* egg imaged with IF and laser scanning microscope based on protocols described in this chapter. Microtubules are shown in red, and DNA and unspecific background staining in green

fertilized egg to 25 µm cells at the midblastula transition [13, 14, 28, 29]. Our main modifications are as follows. First, we used a milder fixation condition that allows faster antibody penetration and deeper structure imaging even in whole-mount embryos. Furthermore, we reduced sample preparation time from ~1 week to ~3 days. We have primarily used this protocol to image specimens from *Xenopus laevis*. However, we also successfully adapted the protocol to obtain immunographs from Axolotl and *Xenopus tropicalis* [15]. An image of a whole-mount *Xenopus* egg with an observable meiotic spindle prepared using the protocol outlined here is shown in comparison with an immunograph of thin-sectioning meiotic egg visualized by Schultze in 1887 (Fig. 1).

2 Materials

2.1 Fixation

1. Methanol fixative: 90% Methanol, 10% 0.5 M EGTA pH 7.8 (adjust pH with KOH).
2. Low FG fixative (modified from [27]): 0.25% to 0.50% formaldehyde (from 37% stock), 0.1% glutaraldehyde (from 50% stock), 80 mM PIPES pH 6.8 (adjust pH with KOH), 1 mM $MgCl_2$, 5 mM EGTA, 0.2% Triton X-100.

3. 100% Methanol.
4. No. 5 watchmaker's forceps.
5. Scalpel.
6. Orbital shaker or nutator.

2.2 Rehydration

1. TBS: 10 mM Tris–HCl, pH 7.4, 155 mM NaCl (make as a 10× stock and add 0.65 g/L of NaN_3 to inhibit bacterial growth). Store at 4 °C.
2. 100% Methanol.
3. Orbital shaker or nutator.

2.3 Hemisecting

1. TBS.
2. Agarose cushion.
3. No. 5 watchmaker's forceps.
4. Scalpel.

2.4 Bleach

1. Bleach solution [30] (make fresh): 1% H_2O_2, 5% formamide, 150 mM NaCl, 16 mM sodium citrate, pH 7.0 (adjust pH with NaOH).
2. TBS.
3. Orbital shaker or nutator.

2.5 Stain

1. TBSNB [27] (make fresh): TBS, 0.1% Igepal CA-630, 1% BSA (prepare as a 10% stock and store at −20 °C), 2% fetal calf serum (FCS). Store at 4 °C for up to a week.
2. Tubulin labeling: Antibodies can be purchased pre-labeled (e.g., Alexa 488—Cat. # 322588, Thermo) or being labeled with dyes (e.g., APEX™ Antibody Labeling Kits, Invitrogen). We have successfully used Alexa 488, 547, or 647—longer wavelengths will result in less background fluorescence. Followings are some suggestions:
   (a) α-Tubulin monoclonal antibody (B-5-1-2) (T6074, Sigma)—for microtubules
   (b) γ-Tubulin monoclonal anti-γ-tubulin antibody (T5326, Sigma)—stains centrosomes
3. DNA staining: The chosen dye should be spectrally separable from the tubulin label:
   (a) TO-PRO-3 (far red) (Invitrogen)
   (b) YO-PRO-1 (green) (Invitrogen)
4. TBS.
5. Tin foil.
6. Orbital shaker or nutator.

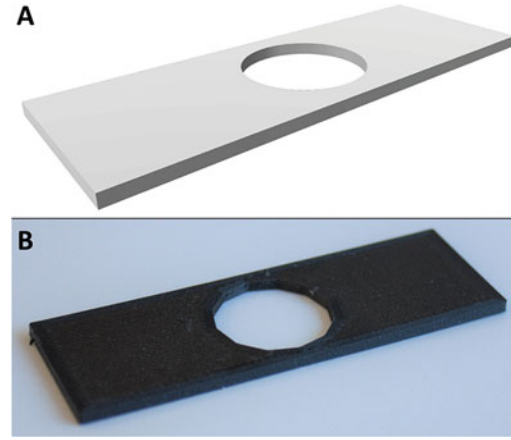

Fig. 2 A 3D printed mounting slide. (**a**) A 3D drawing of the mounting slide. (**b**) 3D printed Onyx (Markforged) slide, which is compatible with Murray's clear

2.6 Clear

1. Murray's clearing solution: 2:1 Benzyl benzoate:benzyl alcohol (BB:BA). Store at room temperature.
2. 100% Methanol.

2.7 Mount

1. Murray's clearing solution.
2. A 3D printed or machined cover slide with opening (Fig. 2 and *see* **Note 1**).
3. 25 mm Circle coverslips (No. 1.5, VWR).
4. Mini block heater (VWR) (*see* **Note 2**).
5. Confocal laser scanning microscope—e.g., LSM Zeiss 880.

3 Methods

We refer the readers to detailed protocols regarding collection of oocytes, eggs, testes, fertilization, and dejellying in previous publications [31–34].

3.1 Fixation

Fixation of the *Xenopus* embryo for IF microscopy requires a compromise between optimal preservation of cellular structures, antibody reactivity, and permeability of the antibody so that it can reach the target structures. Adapting from Becker and Gard [27], we recommend two different fixation conditions. The simpler and easier-to-reproduce procedure is the fixation with methanol with EGTA. The fix is performed at room temperature to prevent microtubule depolymerization. EGTA is added to capture Ca^{2+} ions, which might be released from the endoplasmic reticulum upon fixation and could lead to microtubule disassembly. The methanol fixation works well for metaphase and early anaphase/telophase microtubule. However, this soft fixation with methanol

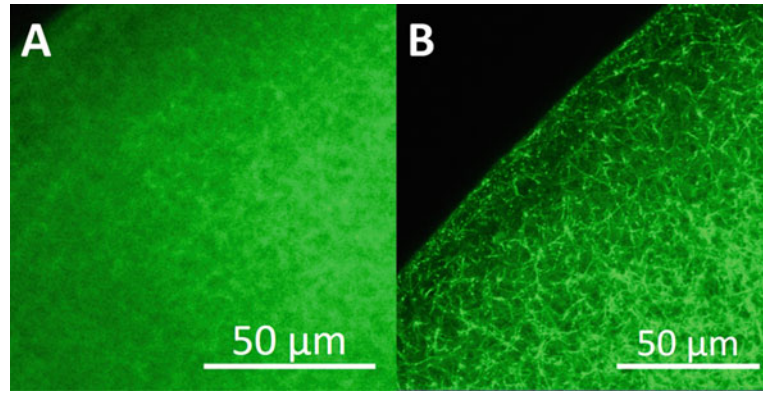

Fig. 3 Comparison of background microtubules (green) in the meiotic egg fixed with methanol or low FG. (**a**) Egg background microtubule organization in methanol fixation is poorly conserved. (**b**) The same microtubule network is clearly apparent with low FG fixation

often leads to deformed embryos. For instance, when samples are treated with methanol around the midblastula transition (MBT), the embryos often disassemble during sample handling. Lastly, some microtubules, e.g., the background network in the meiosis II egg, or late interphase/telophase asters, are only poorly preserved and hard to visualize with this fixation (Fig. 3a). We have obtained the best possible images with the low formaldehyde glutaraldehyde (low FG) fixation. Compared to the Gard protocol we omit the addition of the microtubule stabilizer paclitaxel. We were worried about the formation of new microtubules during the fixation process. With low FG fixation the background microtubules in the *Xenopus* egg can be nicely visualized (Fig. 3b). Furthermore, low FG-fixed embryos retain their shape better and stay intact even around the MBT. The disadvantage of this fix is that it requires more effort and results are harder to reproduce. Over-fixation can prevent deep antibody penetration. We therefore recommend cutting embryos with a scalpel every few minutes to check the progress while fixing. The embryos are fixed just right when the entire inside of the embryo is solid, and the entire cutting surface remains even. As soon as this is achieved, we transfer the embryos into MeOH/EGTA for postfixation of at least 24 h. Samples can be stored at 4 °C for months in methanol.

Methanol Fixation

1. Collect and submerge up to 20 dejellied embryos/eggs/oocytes in ~1.8 mL MeOH/EGTA solution in a 2 mL Eppendorf tube.
2. Gently flick the tube so that the specimen does not clump together.
3. Place the Eppendorf tube sideway on the shaker and leave it shaking gently at room temperature for at least 24 h. Continue to **step 6**.

Low FG Fixation

1. Collect and submerge 40 dejellied embryos/eggs/oocytes in low FG solution in a petri dish (*see* **Note 3**).
2. Place the dish on a shaker, gently shaking.
3. Check the eggs continuously (every few minutes) by cutting embryos with a scalpel in halves until they just turn solid.
4. The extent of proper fixation is rather specific in low FG method, e.g., too little then the microtubules are not preserved, or too much then the system becomes impenetrable to antibodies. The exact timing of low FG fixation can vary significantly depending on the (1) formaldehyde stock, (2) glutaraldehyde stock, and (3) exact fixation temperature.
5. Once the desired consistency is achieved, replace low FG solution with MeOH/EGTA for postfixation. Continue to **step 6**.

From this point onwards, both methods are the same:

6. Incubate the sample for at least 24 h in MeOH/EGTA while gently rotating on a nutator.
7. Replace the solution with 100% methanol.
8. STOP POINT store at 4 °C ad infinitum.

3.2 Rehydration

Antibodies are not methanol compatible. Prior to labeling, the sample needs to be rehydrated in a water-based solution. The rehydration process should be done gradually via multiple changes of TBS/MeOH solution with increasing TBS volume concentration to avoid bubble formation and disintegration of specimen. This is particularly important for methanol-fixed embryos.

1. Rehydrate embryos/eggs/oocytes in a sequence of 25%, 50%, 75%, and 100% TBS/methanol. Sample should be incubated in each solution for at least 30 min.
2. Store the samples in TBS.
3. SHORT-TERM STOP POINT.

3.3 Hemisecting Xenopus Oocytes and Eggs (Optional)

Hemisecting is useful because the large size of *Xenopus* oocytes, eggs, and embryos could hamper the penetration of antibodies—particularly after fixation with aldehyde. In addition, properly mounted hemisected oocytes and eggs allow visualization of regions of interest buried deep inside that would otherwise be inaccessible for short-working-distance objectives. When fixing later stage embryos with methanol, whole mount is highly recommended (*see* **Note 4**).

Routinely, we hemisect embryos/eggs/oocytes with a sharp scalpel prior to bleaching or staining; hence we retain the ability to tell the difference between the animal and vegetal poles (*see* **Note 5**).

1. Transfer the embryos to a petri dish with an agarose bed filled with buffer (100% TBS). The soft agarose bed helps to keep embryos intact during hemisecting.
2. Hemisect embryos along the desired axis with a scalpel.
3. After cutting, return oocytes to Eppendorf tubes filled with TBS.
4. SHORT-TERM STOP POINT.

3.4 Bleach Embryos

The pigments of *Xenopus* oocytes, eggs, and embryos attenuate laser illumination and obscure fluorescence. Pigmentation of fixed *Xenopus* oocytes can be eliminated by bleaching them with a solution of H_2O_2 (*see* **Notes 6** and **7**). Once bleached, the animal and vegetal hemispheres of the embryos/eggs/oocytes are often almost indistinguishable. If one works with albino embryos, this step is unnecessary (*see* **Note 8**).

1. Carefully aspirate the TBS.
2. To minimize accidental aspiration of samples, use a micropipette tip (preferably 200 μL) to reduce the bore of the Pasteur pipet tip.
3. Place the samples in the bleaching solution for a few hours or overnight. The required time varies depending on the number of samples in the bleaching solution and the amount of pigmentation. We suggest incubating the samples with the tubes on their sides on the shaker at room temperature (Fig. 4).
4. Carefully aspirate the bleaching solution.
5. Wash twice for ~15 min with TBS.
6. SHORT-TERM STOP POINT.

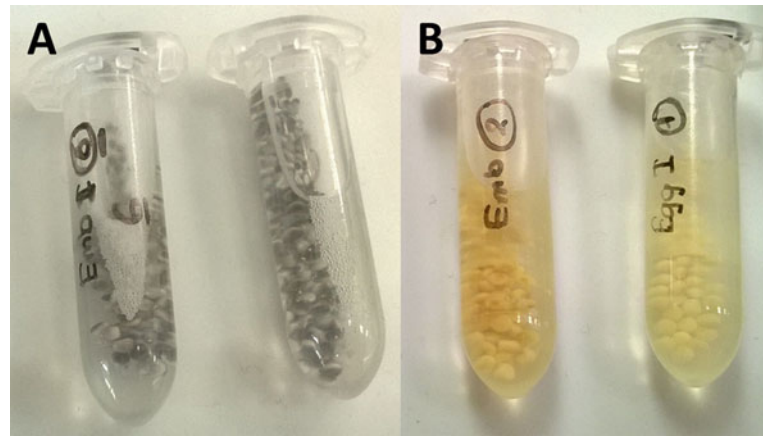

Fig. 4 Embryos before and after bleaching. (**a**) Post-rehydration, pre-bleaching embryos incubated in TBS. (**b**) Post-bleaching embryos incubated in TBS

3.5 Stain with Antibodies and DNA Dyes

Processing *Xenopus* embryos/eggs/oocytes for IF requires substantial antibody incubation time, with corresponding increase in the duration of intermediate washes, to allow the desired penetration (*see* **Note 9**). After staining, we dehydrate the prepared specimen with methanol to be able to transfer the embryos into the hydrophobic Murray's clear (*see* **Note 10**).

Multiple antibodies against cytoskeletal proteins with appropriate fixation conditions and working dilutions are listed in Table 1 in Becker and Gard's paper [27]. In this document, we only discuss antibodies against microtubules and dyes for DNA staining. We typically start with a 1:200 dilution of a 1 μg/μL antibody stock solution. The dilution can later be adjusted to either minimize background or increase brightness (*see* **Note 11**).

Here we exemplify the protocol with an Alexa-488-labeled α-tubulin antibody and the DNA dye TO-PRO-3 (*see* **Note 12**).

1. Submerge ~10 embryos in approximately 400 μL TBSNB.
2. Incubate primary labeled antibody (Alexa-488-labeled tubulin antibody) at 1:200 dilution (stock 1 μg/μL) in TBSNB.
3. Cover in tin foil and lay it down sideways on the shaker.
4. Incubate at 4 °C while gently shaking for at least 12 h (better 24 h).
5. Wash in TBSNB for at least 24 h (better 48 h) at 4 °C with a few times replacing buffer.
6. Keep the tube on gentle shaking mode and covered in tin foil.
7. Incubate in TBSNB for 30 min with TO-PRO-3 at 5 μM concentration.
8. Wash in TBSNB for 1 h.
9. Wash twice in TBS for 10 min.

3.6 Clear Embryos for Confocal Microscope

The yolk renders embryos/eggs/oocytes opaque and prevents visualization of structures no more than a few micrometers below the cell surface. The refractive index of Murray's clear closely matches that of yolk, thereby rendering *Xenopus* oocytes and eggs nearly transparent [26] (*see* **Note 13**).

1. Dehydrate samples in methanol in two changes.
2. Wash with MeOH for at least 15 min.
3. Repeat MeOH wash for 15 min for at least three times.
4. Aspirate the methanol.
5. Add ~1 mL Murray's clear to the samples. Allow them to clear and sink slowly to the bottom of the tube as they are infiltrated by Murray's clear solution (taking about 5–15 min). Do not stir the embryos in the solution.

6. When embryos have sunk, carefully remove the supernatant (Murray's clear mixed with methanol) and replace the top of vial with new Murray's clear (*see* **Note 14**).
7. At this point, the embryos should become transparent and somewhat difficult to identify in the solution (*see* **Note 15**).
8. SHORT-TERM STOP POINT (*see* **Note 16**).

3.7 Mount

Because of their size and physical properties, mounting *Xenopus* embryos/eggs/oocytes for high-resolution microscope poses some challenges. Therefore, they must be securely mounted between the coverslips to prevent movement during image collection period. We suggest orienting them so that the region of interest is at the closest proximity to the objective. Imaging with inverted microscopes is preferable. With upright microscopes the thickness of the mounting slide might need to be adjusted. For mounting whole oocyte/eggs/embryos, we suggest using double-sided chambers which have the dimensions of a typical glass and a thickness of 1.2 mm for whole mount or 0.8 mm for hemisected [27], with coverslips attached to both sides. For interested readers, we provide here (Supplementary Material) a 3D printable file of such a double-side chamber which is ready to use for a machine shop or common 3D printing vendors, e.g., https://www.3dhubs.com/. In our hands, Onyx (Fig. 2)—a 3D printable, filament made from nylon with micro-carbon reinforcement—works well, is cost effective, and is easy to obtain.

1. To make the mounting slide (Fig. 5), cut a small piece of parafilm (25 × 40 mm).
2. Place the parafilm beneath the mount; use a scalpel to go around the edge of the hole; cut out a circular piece. Discard the circular part and keep the cutout piece of parafilm.
3. Place the parafilm on top of the mount so that the circular hole and the chamber are aligned. Then place the coverslip on top of the parafilm.
4. Place the assembly on a heating block (~60 °C) for ~10 min with the coverslip facing downward. The heat will melt the parafilm to help glue the glass to the slide.
5. Once the parafilm melts, take the slide off the heating block. Gently push the glass against the mount to ensure sealing any gap. The mounting slide is ready for use.
6. Transfer the embryos (with the Murray's clear solution) onto the appropriate slide.
7. Add more Murray's clear solution until a convex meniscus forms (*see* **Note 17**).
8. Close the open side with a coverslip. Make sure that no air bubbles are trapped.

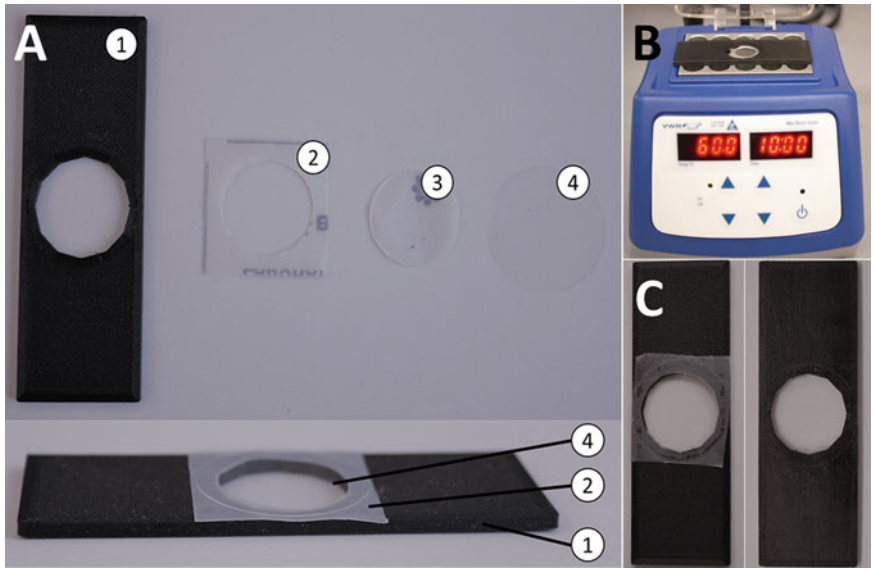

Fig. 5 Assembly of a mounting slide. (**a**) Components include (1) a mounting slide, (2) a cutout piece of parafilm, (3) a circular piece of parafilm—discard, and (4) a coverslip. The assembly is put together in the order from the bottom to the top: slide, parafilm, and coverslip. (**b**) Place the assembly with the coverslip facing downward on a heating block until the parafilm melts. (**c**) The result is a mounting slide with the bottom sealed by a coverslip

9. Gently press down on the coverslip to seat it properly.
10. Aspirate any excess Murray's clear expelled from the well.
11. Slides are immediately ready to view on the confocal microscope or storage for a few days in the dark at room temperature (*see* **Note 18**).
12. SHORT-TERM STORAGE.

How to optimally acquire laser scanning images has been extensively discussed elsewhere [35, 36]. In addition, postprocessing of confocal images is also discussed in detail in reference [37].

To demonstrate the versatility of the protocol we provide a few examples of *Xenopus* oocytes and embryos shown in Fig. 6. We believe for many IF experiments in amphibian systems, the outlined protocols are a useful starting point. A few previously published examples of IF imaging against, e.g., a mitotic regulator—AurkB, a mitotic kinesin—Kif20A, an actin-binding factor—Anln, and a cadherin-binding protein—Sept9 have successfully shown adaptability of these methods to other proteins [38, 39]. Please note that the difficult imaging conditions in amphibian oocytes and embryos might hinder visualization of low-abundant proteins. Some hints on whether imaging is possible can be obtained from proteomics studies with estimation of protein abundance in eggs and embryos [18, 40]. The proteins we were successfully able to image have estimated expression levels in the egg of at least 20 nM.

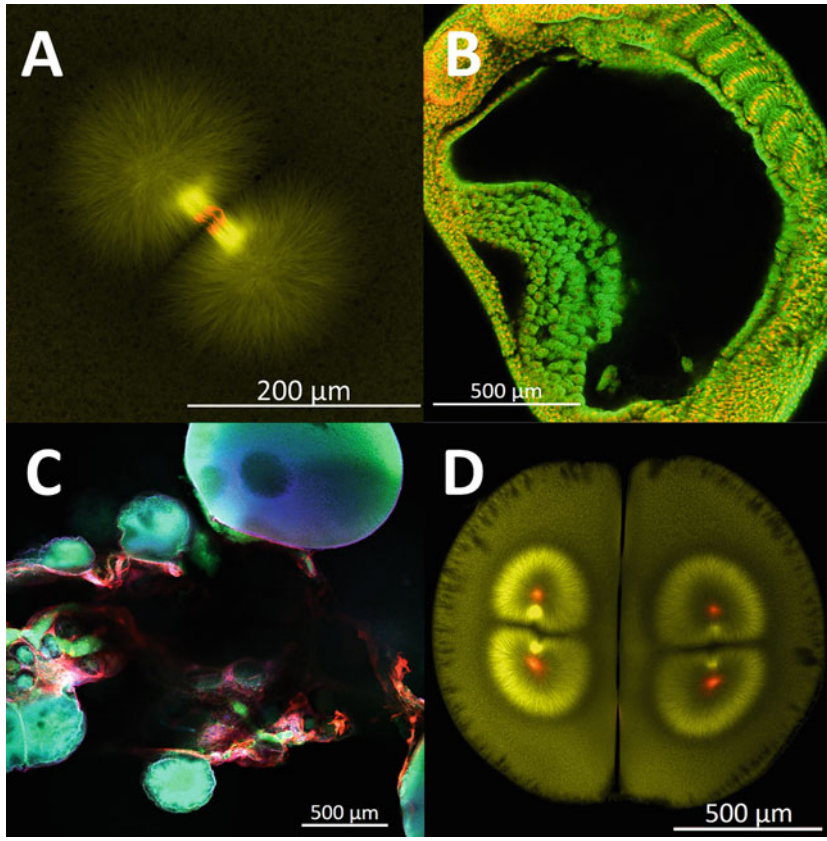

Fig. 6 Exemplary IF images of *Xenopus* oocytes and embryos. (**a**–**c** were fixed in methanol, and **d** in low FG.) (**a**) A close-up of the mitotic spindle in early anaphase from a one-cell-stage embryo. Chromosomes are shown in red, and microtubules in yellow. (**b**) An image of a methanol-fixed embryo at stages 32–34. Microtubules are shown in green, and DNA in red. (**c**) IF of a *Xenopus* ovary showing oocytes at various stages of growth. DNA and unspecific background is stained in green, microtubules are shown in blue, and intermediate filaments in red. (**d**) A two-cell embryo during anaphase/telophase. α-tubulin and γ-tubulin stainings are shown in yellow and red, respectively

4 Notes

1. A 3D model file is available in Supplementary Material.
2. Set heating block to ~60 °C.
3. We suggest to start with more specimens than needed because low FG fixation requires a few extra specimens for consistency check throughout fixation progress. We suggest using 2–3 specimens per inspection.
4. Hemisecting, however, may be unfavorable in later embryonic stages prior to gastrulation. At this point, embryos contain many cells that are loosely intact, and hemisecting increases their chance of falling apart.

5. We prefer to perform the cutting on an agarose cushion to hold the oocyte/egg/embryo in place, and to support the embryo leading to a cleaner cutting surface. Nevertheless, for simplicity, cutting directly on the plastic surface of a petri dish works reasonably well.
6. Perform the bleaching step prior to adding fluorophore to prevent bleaching of the fluorophore.
7. Peroxide bleach is very reactive with clothing and causes painful chemical burns on contact with skin. Gloves and protective glasses should be worn, and care taken when using bleach. In addition, be aware of whether the used antibodies/epitopes are compatible with peroxide bleach.
8. In an unbleached, non-albino embryo, animal hemisphere is pigmented while vegetal hemisphere is not. There is no easy procedure to distinguish the two hemispheres in a bleached or albino embryo.
9. To reduce time, we prefer to work with directly labeled primary antibodies. Directly labeled antibodies can be obtained commercially, or generated with amine-reactive dyes (e.g., APEX™ Antibody Labeling Kits, Invitrogen) [41]. In our experience, antibody incubation times of 12–24 h and washes of 24–48 h are satisfactory for either hemisected or whole-mount samples.
10. It is important to use dry methanol. If water is introduced into the clearing solution the specimen will not become fully transparent.
11. Diluted primary antibodies can be reused several times.
12. The most commonly used dyes for DNA such as Hoechst and DAPI are not compatible with Murray's clear. We have used TO-PRO-3 (far-red) and YO-PRO-1 (green) and observed good results for DNA staining of mitotic chromosomes. However, in our experience staining of nuclei in interphase is more finicky and harder to reproduce.
13. Murray's clear dissolves many plastics, including polystyrene and cellulose acetate [27]. Polypropylene tubes (e.g., Eppendorf tubes and some Falcon tubes) are resistant and should be used for all steps utilizing the clear. Finally, benzyl benzoate is an eye and skin irritant and should be handled with care.
14. The methanol-filled samples are lighter than Murray's clear; hence they are floating. Over time the methanol exchanges with Murray's clear and the samples will sink to the bottom. The methanol will stay close to the surface and can be easily removed.
15. If the samples remain cloudy still, they may not be completely dehydrated. Pass them through a few changes of methanol to

remove clearing solution and the remaining aqueous buffer; then re-clear with Murray's clear.

16. We observed that the sample might regain some opacity if stored in Murray's clear for too long.
17. At this point it might be advantageous to reorient the embryos as desired for imaging under a dissecting microscope.
18. Handle with care to keep the specimen in place.

Acknowledgments

We thank Sean Megason, Angela DePace, Evangelos Gatzogiannis, Mike Levine, and Laurence Lemaire for usage of their microscopes. Thanks to members of the Wühr Lab for comments on the manuscript. This work was supported by grants GM39565 and 1R35GM128813 from the National Institutes of Health and Princeton University startup funding.

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Chapter 3

Assaying NanoLuc Luciferase Activity from mRNA-Injected *Xenopus* Embryos

Michael D. Sheets

Abstract

The earliest steps of animal development depend upon posttranscriptional events that drive the embryonic cell cycle and guide cell fate decisions. The analysis of post-transcriptional regulatory events has relied upon the use of chimeric reporter mRNAs that encode firefly luciferase fused to potential regulatory sequences. A new and more sensitive luciferase developed recently called NanoLuc has the potential to improve reporter studies and provide new insights into the regulation of embryonic processes. Here I describe how to create and analyze reporter mRNAs encoding NanoLuc luciferase using extracts from microinjected *Xenopus* embryos.

Key words *Xenopus* embryos, mRNA microinjection, NanoLuc, Luciferase

1 Introduction

Developmental biologists have long embraced the use of reporter technologies for their studies. The myriad applications of these tools have provided significant new insights into the fundamental processes that occur in embryonic cells. For example, firefly luciferase-based reporters have been used extensively to quantitatively monitor spatial and temporal gene expression at both the transcriptional and post-transcriptional levels [1–4]. In addition, fluorescent reporters have been used extensively to follow the temporal and spatial expression and activities of specific proteins [5, 6].

The utility and adoption of the different reporters as experimental tools have largely paralleled technical advances. The widespread use of firefly luciferase was greatly facilitated by the cloning of the luciferase enzyme and development of sensitive instruments for measuring luciferase activity along with the establishment of assay conditions and substrates that produced prolonged and easily detectible light signals [7, 8]. The identification of fluorescent proteins coupled to the creation of tools to generate specific protein fusions has revolutionized the study of development and

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_3,

significantly advanced our knowledge of key embryonic processes [5, 6]. To further expand the utility of luciferase reporters a new luciferase, called NanoLuc, was developed using directed evolution of an enzyme from the deep-sea shrimp *Oplophorus gracilirostris* [9]. NanoLuc is a small monomeric protein that utilizes a novel substrate to generate stable luminescence as well as a higher specific activity compared to other luciferase enzymes. This reporter and its unique properties offer numerous advantages for developmental biologists. Particularly, the increased sensitivity of NanoLuc compared to firefly luciferase provides the potential to analyze the activity of reporter mRNAs at concentrations approaching the physiological levels present in embryonic cells [10]. In the following sections I describe how to create and analyze reporter mRNAs encoding NanoLuc luciferase using extracts prepared from microinjected *Xenopus* embryos.

2 Materials

2.1 Plasmid for Creating Chimeric NanoLuc mRNAs

1. To generate mRNAs in vitro for analysis requires a plasmid containing the NanoLuc coding region downstream of a T7 promoter. The plasmids generated by Promega all lack a T7 promoter for generating mRNAs in vitro. To address this issue we created pT7-NanoLuc from pNL1.1 (Fig. 1). In addition to the T7 promoter the XbaI restriction site downstream of the NanoLuc coding region facilitates the cloning of 3′UTR fragments for analysis.

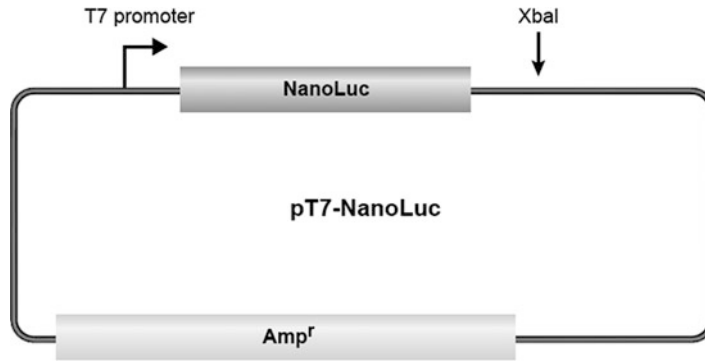

Fig. 1 The pT7-NanoLuc plasmid for creating NanoLuc mRNAs in vitro. The pNL1.1 plasmid (Promega) was modified by cloning the promoter for T7 RNA polymerase upstream of the NanoLuc coding region. An XbaI restriction site is present 3′ of NanoLuc and can be used for cloning DNA fragments that encode 3′UTRs for analysis

2.2 In Vitro Transcription to Generate NanoLuc mRNAs

1. Plasmid for transcription linearized with appropriate restriction enzyme.
2. T7 RNA polymerase.
3. 10× Transcription buffer: 400 mM Tris–HCl, 60 mM $MgCl_2$, 10 mM DTT, 20 mM spermidine, pH 7.9@25 °C.
4. Nucleotide solutions, ATP 100 mM, CTP 100 mM, UTP 100 mM, GTP 20 mM, CAP analog 40 mM.
5. Nuclease-free water.
6. G50 spin columns.
7. Agarose.
8. 5× Tris-borate-EDTA (TBE) buffer (450 mM Tris-borate, 10 mM EDTA).
9. RNA-loading dye (47.5% formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% xylene cyanol, 0.5 mM EDTA).

2.3 Microinjection into Xenopus Embryos

1. Microinjection apparatus.
2. Dissecting microscope.
3. Microinjection dishes with mesh grids to hold embryos in position.
4. Embryo culture media Marc's Modified Ringer's Solution (MMR)—10× stock: 1 M NaCl, 20 mM KCl, 10 mM $MgSO_4$, 20 mM $CaCl_2$, 50 mM HEPES (pH 7.8), 1 mM EDTA. Adjust pH to 7.4, sterilize by autoclaving, and store at room temperature.
5. MMR 0.25× (working solution).
6. MMR 0.25×, 4% Ficoll 400.

2.4 Generating Embryo Extracts for NanoLuc Assays

1. Luciferase Cell Culture Lysis Reagent 5× (Promega).
2. Micro-pestles.

2.5 Assaying Embryo Extracts for NanoLuc Activity

1. Nano-Glo Luciferase Assay Substrate (Promega).
2. Nano-Glo Luciferase Assay Buffer (Promega).
3. Luminometer assay tubes.
4. Luminometer (for example, for single-tube analysis the Turner Designs 2020 or the Berthold Lumat LB9507 instruments work well). In addition, for the analysis of large number of samples there are a variety of luminometers capable of analyzing samples in microtiter plates.

3 Methods

3.1 Generate the Plasmid Template for Transcription

1. Plasmid DNA is linearized by restriction enzyme cutting to create a template for in vitro transcription.
2. The linearized DNA template is purified by extraction with an equal volume of 1:1 phenol/chloroform mixture.
3. The DNA is precipitated from the reaction by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and three volumes of ethanol. Incubate at −20 °C for 60 min.
4. The DNA precipitate is spun out of solution using a microcentrifuge operating at 10,000 RPM (10,600 × *g*), for 15 min at 4°.
5. The supernatant is removed and the DNA pellet is rinsed with 500 μL of cold 70% ethanol and centrifuged for 5 min.
6. Remove the wash, air-dry the pellet, and resuspend it in DEPC-treated water at a concentration of approximately 0.5–1.0 μg/μL.

3.2 In Vitro Transcription to Generate NanoLuc mRNAs [11] (See Note 1)

1. Assemble 20 μL reactions at room temperature in the following order (*see* **Note 2**):

   Nuclease-free water X μL

   10× Transcription buffer 2 μL

   2 μL ATP 100 mM—10 mM final concentration

   2 μL UTP 100 mM—10 mM final concentration

   2 μL CTP 100 mM—10 mM final concentration

   2 μL GTP 20 mM—2 mM final concentration

   4 μL Cap Analog 40 mM—8 mM final concentration

   Template DNA X μL 1 μg

   T7 RNA polymerase 2 μL

   Total reaction volume 20 μL

2. Mix by micropipetting and incubate at 37 °C for 2 h.
3. Add nuclease-free H_2O to the reaction until the volume reaches 100 μL.
4. G50 spin columns are used to remove unincorporated nucleotides, proteins, and salts. Apply the reactions to the column and follow the manufacturer's instructions for sample purification.
5. Measure ultraviolet light absorbance at 260 nm (*see* **Note 3**).
6. Analyze an aliquot of the mRNA on a denaturing agarose gel to evaluate length, integrity, and quality [12, 13] (*see* **Note 4**).

3.3 Microinjection into Xenopus Embryos

1. Generate *Xenopus* embryos and microinject reporter mRNAs using standard protocols [14–17] (*see* **Note 5**).
2. Incubate injected embryos in 0.25MMR 4% Ficoll until the desired stage of development is reached (*see* **Note 6**).

3.4 Generating Embryo Extracts for NanoLuc Assays

1. Examine injected embryos and discard any damage during microinjection.
2. Place healthy embryos in 1.5 mL microcentrifuge tubes.
3. Remove excess culture media taking care not to lyse the embryos.
4. Add 100 μL cell culture lysis reagent to each embryo sample (*see* **Note 7**).
5. Lyse the injected cells with a micro-pestle.
6. Spin the extracts at 4 °C for 10 min at 10,000 RPM (10,600 × *g*) in a microfuge.
7. Transfer the supernatant extracts to a new microcentrifuge tube while leaving behind the particulate pellets and yolk.
8. Assay extracts for NanoLuc activity immediately or store extracts at −80 °C indefinitely (*see* **Notes 8** and **9**).

3.5 Assaying Embryo Extracts for NanoLuc Activity

1. Thaw extracts on ice or if generated fresh store on ice until use (*see* **Notes 8** and **9**).
2. Prepare a working solution of the NanoLuc substrate by diluting the concentrated substrate 1/50 in NanoLuc assay buffer (*see* **Note 10**).
3. Prepare samples by mixing 50 μL of substrate with 50 μL of extract and incubating at room temperature for 3 min protected from light (*see* **Note 11**).
4. Transfer samples to luciferase assay tubes and assay for NanoLuc activity using a luminometer (*see* **Notes 12** and **13**).

4 Notes

1. Follow precautions for working with mRNA to avoid RNase contamination that could degrade the mRNA. Wear gloves at all times and use only nuclease-free tubes and reagents.
2. Reactions are typically 20 μL but can be scaled up if large amounts of mRNA are needed.
3. Calculate mRNA concentration, with one A260 unit being equivalent to an mRNA concentration of 40 μL/mL.
4. Mix 0.2 μg mRNA sample with RNA-loading dye. Denature the mRNA sample by heating at 65 °C for 5 min. Load sample onto a 1% agarose gel (TBE buffered) and electrophorese. Visualize RNA by staining the gel with ethidium bromide.
5. Concentrations of mRNAs in samples for injection will vary depending upon individual mRNA and specific application. Typically we use samples that contain 6 attomoles/nL (1 pg/nL) mRNA and inject 1–10 nL per embryo [14–16].

6. Embryos can be cultured at lower temperatures approaching 13 °C to slow development when collecting samples for time-course experiments.
7. The volume of cell culture lysis buffer is typically 100 μL/embryo but the volume can be varied to accommodate different signal intensities.
8. Extracts can be assayed immediately after production or stored at −80 °C and assayed when convenient.
9. We have not observed a reduction of activity upon freezing and thawing extracts multiple cycles.
10. Each assay requires 50 μL of substrate. The prepared substrate is kept at room temperature protected from light until use.
11. Assay each extract in triplicate to provide technical replicates. Analyze multiple dilutions of each extract to ensure that readings are in the linear range of the assay conditions and the luminometer. Prepare dilutions of extract with cell culture lysis buffer. Once prepared the cell culture lysis buffer can be stored at −20° until use.
12. Occasionally negative control extracts from uninjected embryos produce a background signal (>1000 RLUs). In such cases freezing and thawing extract often eliminate the background.
13. Although this chapter focuses on analyzing NanoLuc activities in *Xenopus* embryos similar approaches can be applied to *Xenopus* oocytes, eggs, and embryos of other model organisms used by developmental biologists.

Acknowledgments

Work in the Sheets lab was supported by NIH grants (R21HD076828, R01HD091921).

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Chapter 4

Reconstitution of Intracellular Calcium Signaling in *Xenopus* Egg Extracts

Alexander A. Tokmakov and Ken-Ichi Sato

Abstract

Generation of calcium signal in the cytoplasm of fertilized or parthenogenetically activated eggs has been extensively studied in the intact eggs of several biological species. Calcium transient was found to elicit a plethora of biochemical and cellular events in these cells. Remarkably, intracellular calcium signaling can also be reconstituted in cell-free environment. In this chapter, we describe the methods that allow reconstitution, detection, and quantification of the calcium signal in cell-free extracts of *Xenopus* oocytes and eggs.

Key words Calcium, Signal transduction, *Xenopus laevis*, Egg, Oocyte, Cell-free extracts

1 Introduction

1.1 Calcium Signal in Living Xenopus Eggs

Oocytes and eggs of the African clawed frog *Xenopus laevis* have been widely used to study meiotic progression and fertilization. In fact, many of the control mechanisms that operate in maturing oocytes, fertilized eggs, and early embryos have first been established in the frog model [reviewed in 1–4]. Important findings concerning involvement of calcium in egg fertilization, activation, and exit from meiosis have been obtained using *Xenopus* eggs. Generation of calcium wave or calcium oscillations in the cytoplasm of fertilized or activated eggs was found to be an early universal event of egg activation detected in all biological species studied. Significant differences have been observed between generation pathways, spatiotemporal patterns, and intracellular mediators of the calcium signal in the eggs of different species [reviewed in 5–7]. The calcium signal in fertilized *Xenopus* eggs represents a single calcium wave that propagates rapidly from the sperm entry point through the whole cytoplasm. Calcium concentration in fertilized *Xenopus* eggs increases about tenfold from the resting level of about 100–200 nM to above 1 μM within 5 min, due to the calcium release from intracellular stores, and returns to the

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_4,

pre-activation level in about 20 min after fertilization [8, 9]. To simulate fertilization-induced calcium release in *Xenopus* eggs, parthenogenetic egg activation has often been implemented. It was found that *Xenopus* eggs can be activated by the treatment with calcium ionophores. The ionophore-induced calcium influx can mimic the fertilization-initiated calcium transient in eggs, leading to complete egg activation. In *Xenopus* eggs, not only the ionophores, but also electrical shock, needle pricking, and hydrogen peroxide, can cause egg activation that is mediated by intracellular calcium release [10]. The intracellular calcium signaling during *Xenopus* egg fertilization and parthenogenetic activation was extensively studied in the intact cells.

1.2 Calcium Release in Cell-Free Extracts

In addition, cell-free oocyte and egg extracts were employed to facilitate the studies of egg activation and intracellular calcium release. The cell-free extracts of *Xenopus* oocytes and eggs can recapitulate a number of cellular processes, such as meiotic exit, nuclear reprogramming, mitotic cell cycle, and apoptosis. As a model system, the extracts have significant advantages against cells. Large volumes (several milliliters) of synchronized homogenous extracts can be obtained from oocytes and eggs of a single frog, and the compounds of various chemical nature can be directly introduced into the extracts, making easier the dissection of multiple signaling mechanisms. Importantly, despite the lack of the intact cell plasma membrane and ordered compartmentalization, *Xenopus* egg extracts can be successfully used to study the calcium signal as well as the events, which take place upstream and downstream of calcium release during egg activation. Delineation of the molecular events, leading to the calcium release in fertilized or parthenogenetically activated *Xenopus* eggs, has been successfully carried out by several groups, including ours, using intact eggs and cell-free extracts [reviewed in 11–13]. These studies established the sequential activation of *Xenopus* Src family kinase, PLCγ, and IP3 receptor of the endoplasmic reticulum (ER) as the early events of the fertilization-induced egg activation preceding calcium signal (Fig. 1). Inhibitors of Src kinase (PP1 and PP2), PLCγ (U-73122), and IP3 receptor (heparin) were found to block the release of calcium. In addition, hydrogen peroxide or catalytically active Src kinase were shown to initiate, via the same cascade, a low-magnitude calcium signal in *Xenopus* egg extracts [14]. It was suggested that propagation of the calcium wave through the egg cytoplasm involves, in addition to the abovementioned IP3-induced calcium release, a calcium-dependent calcium release mechanism [15]. Of note, IP3-regulated calcium stores are sensitized during meiotic oocyte maturation due to ER remodeling into the large clusters, so that IP3 receptors within the clusters have a higher sensitivity to IP3 than those in the non-clustered reticular ER [16, 17]. As a result, the dynamics of calcium release observed

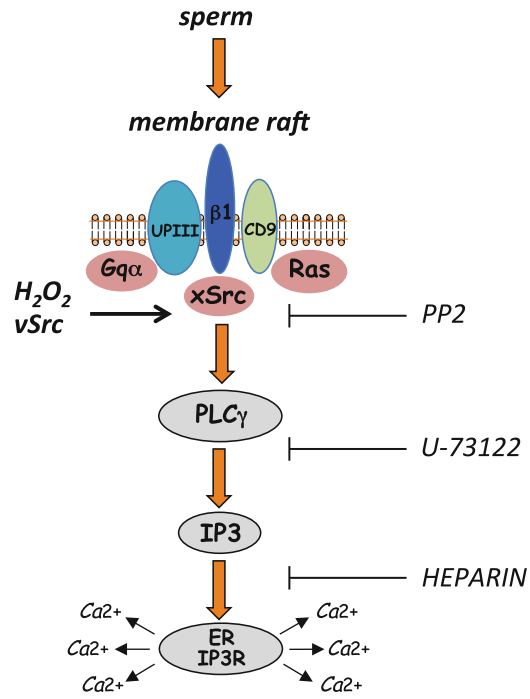

Fig. 1 A model for sperm-, H_2O_2-, Src-, and IP3-induced calcium release in *Xenopus* egg extracts

in *Xenopus* oocytes and eggs after IP3 injection are significantly different [16, 18].

1.3 Calcium Signaling in a Reconstituted System

In spite of disruption of plasma membrane-associated signaling compartments during extract preparation, *Xenopus* egg extracts can be successfully used to study early signaling events that occur upstream of the calcium release during fertilization-initiated egg activation. It has been demonstrated that the fertilization-induced calcium signal originates at the plasma membrane by sperm interaction with a putative, still unidentified egg receptor(s) located in the membrane rafts, a low-density detergent-insoluble membrane fraction of eggs [19]. The plausible candidates for the egg receptor at fertilization include uroplakin III, integrin β1, and CD9 (Fig. 1). In combination with the CSF-arrested extracts, the activated egg rafts can recapitulate early events of egg activation. This becomes possible due to complementation of the sperm-induced molecular signaling machinery present in the egg rafts with the constituents of signal transduction system localized in the extracts. Egg rafts incubated with sperm or hydrogen peroxide were found to induce Src-dependent phosphorylation of PLCγ and calcium transient in the extracts of unfertilized *Xenopus* eggs [20]. Notably, the calcium transient initiates a plethora of molecular and cellular events in the eggs, such as CSF and MPF inactivation, activation of PKC, and cell

cycle transition [reviewed in 7]. Some of the events downstream of the calcium signal can also be detected and studied in the reconstituted cell-free system [13]. In more detail, the events of fertilization-induced calcium signaling in *Xenopus* eggs and egg extracts are described elsewhere [6, 7, 11]. The experimental protocols allowing reconstitution of intracellular calcium signaling in cell-free extracts of *Xenopus* oocytes and eggs are presented below.

2 Materials

1. Frogs: Adult wild-type *Xenopus laevis* female frogs that have never laid eggs before or have not laid eggs for at least half a year are used in the experiments (*see* **Note 1**). The frogs are maintained in dechlorinated tap water at the ambient temperature of 18–21 °C in a light-controlled room with a 12-h-day period.
2. Injections: 1 mL syringes and 27 gauge needles.
3. Priming solution: 100 U/mL of pregnant mare serum gonadotropin (PMSG) in sterile 0.15 M NaCl saline solution; store at 4 °C.
4. Anesthetizing drug: Ethyl 3-aminobenzoate methanesulfonate, MS222, f.c. 2 mg/mL.
5. Ovulation-inducing solution: 2500 U/mL Human chorionic gonadotropin (hCG, Teikoku Zoki, Japan) in sterile 0.15 M NaCl saline solution; store at 4 °C.
6. Oocyte isolation buffer: OR-2 solution containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.6 (*see* **Note 2**).
7. Collagenase solution: 10 mg/mL Solution of collagenase (280 U/mg, Wako, Japan) in OR-2 oocyte isolation buffer.
8. Egg isolation buffer: DeBoer's solution (DB) containing 110 mM NaCl, 1.3 mM KCl, and 0.44 mM $CaCl_2$, adjusted to pH 7.2 by addition of $NaHCO_3$ (*see* **Note 2**).
9. Cysteine solution: 2% Cysteine in DB buffer adjusted to pH 7.8 with 10 N NaOH.
10. Collecting glass tubes # 3-00-203-G/X (Drummond, USA).
11. A dual-stage glass micropipette puller PB-7 (Narishige, Japan).
12. Silicone oil SH-550 (Nacalai Tesque, Japan).
13. A three-axis coarse micromanipulator MM-3 (Narishige).
14. A stereomicroscope MZ8 (Leica Microsystems, Germany).
15. A pulse-directed Nanoject II nanoliter injector system (Drummond).

16. A stainless mesh: 0.8 mm grid (*see* **Note 3**).
17. Extract buffer (XB): 100 mM KCl, 1 mM $MgCl_2$, 50 mM sucrose, 10 mM potassium HEPES, pH 7.7.
18. Protease inhibitors: Stock solution containing 10 mg/mL each leupeptin, pepstatin, and chymotrypsin in DMSO. Store in 50 μL aliquots at −20 °C.
19. Cytochalasin B: Stock solution of 10 mg/mL cytochalasin B in DMSO. Store in 50 μL aliquots at −20 °C.
20. Calcium indicators: Stock solutions of 1 mM Fura-2, Fura-2AM, and Fluo-8AM in DMSO; store in aliquots at −20 °C (*see* **Note 4**).
21. A23187: 10 mM Stock solution in DMSO; store at −80 °C.
22. IP3: 1 mM Stock solution in water, an inducer of calcium release; store at −20 °C.
23. Recombinant Src kinase: $p60^{v\text{-}src}$ (Merck, USA), final concentration 0.2 U/μL, an inducer of calcium release.
24. Hydrogen peroxide: 30% Stock solution, an inducer of calcium release; store at 4 °C.
25. *Xenopus* sperm and membrane rafts: Isolation of *Xenopus* sperm and preparation of the membrane raft fraction from activated eggs are described in detail in the accompanying chapter (Sato and Tokmakov, this volume).
26. Heparin: Final concentration 1 mg/mL, an antagonist of IP3 receptor, inhibitor of calcium release.
27. Src kinase inhibitors PP1 and PP2: 10 mM Stock solutions in DMSO; store at −80 °C (*see* Note **5**).
28. PLCγ inhibitor U-73122: 5 mM Stock in DMSO; store at −80 °C (*see* **Note 6**).
29. SZX16 stereo zoom microscope equipped with a hydrogen lamp (Olympus, Japan).
30. High-frame digital microscope CCD camera DP73 (Olympus).
31. Wide-angle objective SDF PLAPO 1xPF (Olympus).
32. CCD interface U-TV0.5XC-3 (Olympus).
33. CellSens Standard software for signal imaging and processing (Olympus).
34. IX83 imaging system (Olympus).
35. High-frame digital CCD imaging ARGUS/HISCA system (Hamamatsu Photonics, Japan).

3 Methods

3.1 Isolation of Oocytes and Eggs

Isolation of oocytes and eggs is carried out at the ambient temperature of 18–21 °C. The cells collected from a single animal can be considered as a separate oocyte/egg batch. For reproducibility concerns, it is not recommended to mix the cells obtained from different animals.

3.1.1 Isolation of Eggs

1. Prime the frogs by subcutaneous injection of 40 U PMSG into the dorsal lymph sac 5–10 days before egg isolation.
2. Inject 500 U of hCG per a frog to induce ovulation of mature fertilization-competent eggs. Ovulation starts in 8–10 h after the hCG induction.
3. Squeeze the eggs into a plastic Petri dish filled with DB buffer and wash them thoroughly to remove any debris. Several milliliters of eggs can be obtained from one animal.
4. Transfer the eggs into a 50 mL plastic tube and remove excess buffer.
5. Remove the jelly layer covering each egg by incubation with the twofold volume of 2% cysteine solution for 3–8 min. Eggs should be gently agitated during incubation (*see* **Note 7**).
6. Wash the eggs extensively with DB buffer and remove excess buffer.
7. Put ovulated frogs back into a water tank. The animals can recover and ovulate repeatedly within several months (*see* **Note 1**).

3.1.2 Isolation of Oocytes

1. Prime the frogs with PMSG as described above.
2. Put the animals into a small water tank containing the anesthetizing drug MS222 for about 15 min.
3. Place the anesthetized animals on ice and slaughter them by rapid decapitation.
4. Surgically remove the ovaries using forceps and small surgical scissors.
5. Place the ovaries into a plastic Petri dish filled with OR-2 solution and manually sever them into the clamps of 50–100 oocytes with the blunt-end forceps.
6. Transfer ovary clamps into a 50 mL plastic tube and wash thoroughly with OR-2 to remove blood and debris.
7. Remove excess buffer, add an equal volume of collagenase solution to release oocytes from the ovary pieces. This treatment also removes the layer of follicle cells surrounding each oocyte.

8. Incubate the ovary clamps with the enzyme for 2 h with gentle shaking at 30–60 rpm (*see* **Note 8**).
9. Wash the oocytes extensively with OR-2 solution and leave them for stabilization over 4 h.
10. Manually select free undamaged defolliculated oocytes of stage VI for following experiments.

3.2 Preparation of Cell-Free Extracts

To prepare the extracts for reconstitution of cell-free calcium signaling, about a thousand of dejellied eggs or defolliculated oocytes (packed volume about 1 mL) should be collected.

1. Wash the eggs or oocytes with the calcium-free DB or OR-2 buffers, respectively.
2. Wash the cells twice in the extract buffer XB.
3. Wash the cells twice in XB, containing protease inhibitors (leupeptin, pepstatin, and chymotrypsin), each at 10 μg/mL.
4. Transfer the cells to a 1.5 mL Eppendorf tube containing XB, 10 μg/mL protease inhibitors, and 100 μg/mL cytochalasin B (*see* **Note 9**). In the case when the extracts are prepared from CSF-arrested eggs, include 2 mM EGTA in the extract buffer (*see* Note **10**).
5. Centrifuge the tube at 500 × *g* for 30 s and remove XB buffer from the top of the packed oocytes/eggs.
6. Centrifuge the tube at 1500 × *g* for 30 s. All XB buffer should be removed from the top of the packed cells.
7. Crush oocytes/eggs by centrifugation at 12,000 × *g* for 15 min at 4 °C. After the centrifugation, three distinct layers will be visible in the tube: dark gray yolk layer at the bottom, gray yellow cytoplasmic layer in the middle, and white lipid layer at the top.
8. Carefully collect the crude cytoplasmic layer and transfer it to a new Eppendorf tube on ice.
9. Subject the cytoplasmic layer to the second clarifying centrifugation under the same conditions as the crushing spin. Some contamination with yolk and lipid fractions cannot be avoided at this stage. The extract will again separate into the three layers, and the cytoplasmic layer is collected and kept on ice until use.
10. Repeat, if necessary, the clarifying centrifugation one more time under the same conditions. The resulting crude cytoplasmic extract should be transparent yellow.
11. Add cytochalasin B, leupeptin, pepstatin, and chymotrypsin to the extract at a final concentration of 10 μg/mL each.

About 200–300 μL of clear cytoplasmic extract can be obtained from 1 mL of packed oocytes/eggs (*see* **Note 11**). The extracts obtained by the procedure of low-speed centrifugation contain a particulate fraction of intracellular calcium stores that can release calcium in response to various stimuli. The extracts also contain free calcium at a level corresponding to intracellular calcium concentration in unstimulated eggs and oocytes (100–200 nM).

3.3 Upload of Calcium Indicators

Both cell-permeable and -impermeable calcium probes can be directly added to the extracts before initiation of calcium signaling.

3.3.1 Upload to Cell-Free Extracts

1. Dilute 1/10 the DMSO stock solution of calcium indicator with distilled water or physiological solution.
2. Add an aliquot of the water-based indicator solution to the crude cytoplasmic extract of oocytes/eggs. The final concentration of the indicator in the extract may range from 1 to 5 μM (*see* **Notes 12** and **13**).
3. Mix the extract gently by pipetting through a tip with a wide opening (*see* **Note 14**).
4. Pre-equilibrate the extract with the probe for at least 10 min before experiments.

3.3.2 Upload into Oocytes and Eggs

Cell-permeable calcium probes, such as Fura-2AM and Fluo-8AM, can be uploaded into *Xenopus* oocytes and eggs before the stage of extract preparation. This approach is recommended when the experiment involves the extracts obtained by cytoplasmic collections from single oocytes and eggs (see the following section).

1. Place dejellied eggs or defolliculated oocytes maintained in the corresponding isolation buffer into a small Petri dish (diameter 35 mm).
2. Add an aliquot of the stock indicator solution to a final concentration of the indicator ~10 μM.
3. Incubate the cells with the probe for 2–4 h in the dark at the ambient temperature of 18–21 °C.
4. Monitor progression of the probe upload by observing oocyte/egg fluorescence at the corresponding Ex/Em.
5. To remove free indicator, wash the cells thoroughly with the corresponding isolation buffer before extract preparation or cytoplasmic collections.

The kinetics of Fura-2 and Fluo-8 upload to *Xenopus* oocytes are presented in Fig. 2. It appears that a strong fluorescent signal of a subsaturation level can be observed after 4-h incubation of oocytes with the probes.

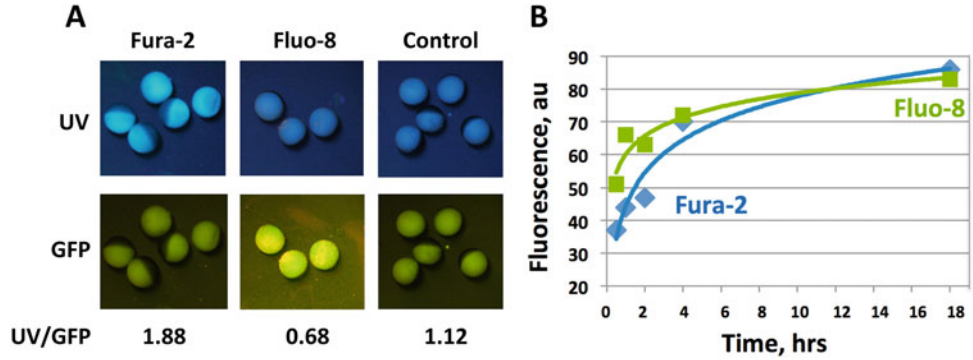

Fig. 2 Upload of calcium indicators into *Xenopus* oocytes. (**a**) The defolliculated oocytes maintained in calcium-free OR-2 buffer were incubated with the cell-permeable calcium indicators Fura-2AM or Fluo-8AM at a final concentration of 10 μM each for 4 h at 21 °C. Fluorescence of Fura-2AM (Ex = 340/380 nm; Em = 510 nm) was observed with the near-UV filters (Ex = 330–385 nm and Em > 420 nm), and fluorescence of Fluo-8 (Ex/Em = 490/520 nm) was registered with the filters optimized for detection of GFP (Ex = 460–495 nm and Em > 510 nm). Ultraviolet-excited signal (UV) and green light-excited signal (GFP) were observed and quantified using fluorescent microscopy. Digits below the panels indicate intensity ratio of UV-excited and green light-excited signals. (**b**) Kinetics of calcium upload into the oocytes. The calcium probes were administered at 10 μM concentration for indicated times

3.4 Cytoplasmic Collections

It is possible to reconstitute intracellular calcium signaling in cell-free *Xenopus* oocytes and egg extracts in the nanoliter-scale experiments using the technique of single-cell cytoplasmic collections. This technique was developed previously for gene expression monitoring in single living *Xenopus* oocytes and eggs [21]. The standard equipment for oocyte microinjection can be used for cytoplasmic collections.

1. Pull the capillary from the specified glass tube using a micropipette puller.
2. Break the glass capillary tip with forceps, so that the tip opening exceeds 50 μm in diameter. Due to a high viscosity of the cytoplasm, the needles for cytoplasmic collections should have a wider tip opening than the needles for microinjection (Fig. 3).
3. Mark the collection needle with a water-resistant laboratory marker pen about 0.5 mm away from the tip opening.
4. Fill the needle with silicone oil.
5. Mount the oil-filled needle on microinjector arm (*see* **Note 15**).
6. To prevent undesirable movement during cytoplasmic collections, place oocytes/eggs on a buffer-immersed stainless mesh positioned in a plastic Petri dish (*see* **Note 3**).
7. Position the capillary for cytoplasmic samplings using a three-axis micromanipulator under microscopic observation.

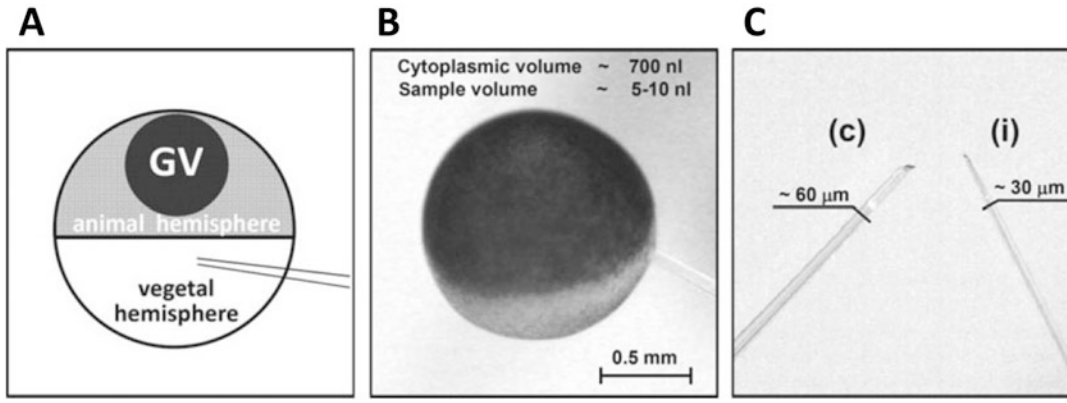

Fig. 3 Cytoplasmic collections from *Xenopus* oocytes. (**a**) Schematic representation of an oocyte and (**b**) its microscopic image with the inserted sampling capillary during the collection of cytoplasmic material. The large oocyte nucleus (germinal vesicle, GV) is located in the animal hemisphere. (**c**) A photograph of the microcapillary tips used for cytoplasmic collections (*c*) and microinjections (*i*). The figure is reproduced from the previous publication [21] with permission from the FEBS Journal (license number 4237460058433)

Cytoplasmic samples should be taken from the near-equatorial vegetal hemisphere of oocytes to avoid hitting the nucleus, which is located in the animal hemisphere (Fig. 3a).

8. Insert the collection needle into a gamete cell to a depth of 300–500 μm, as controlled by capillary marking.
9. Collect the required cytoplasmic volume. The volume of a single cytoplasmic collection may vary from 5 to 50 nL, depending on the duration of a motor pulse in the filling mode of Nanoject II. Usually, 20–50 nL is enough for an experiment involving reconstitution of calcium signaling in the capillary. Multiple cytoplasmic collections can be taken from the same oocyte, considering that the estimated cytoplasmic volume of one oocyte is about 700 nL (Fig. 3b) (*see* **Notes 16** and **17**).

3.5 Initiation of the Calcium Signal

3.5.1 Initiation of the Calcium Signal in Extract Droplets

The calcium signal in *Xenopus* oocyte and egg extracts can be initiated by direct introduction of a solution containing the activator(s) of the calcium-releasing pathway presented in Fig. 1. In the case of reconstituted system, freshly prepared *Xenopus* sperm and membrane rafts from activated eggs should be used.

1. If required, add the inhibitors of calcium release, such as PP1, PP2, U-73122, and heparin, to the extracts 10–30 min before initiation of calcium signaling.
2. Upload the specified calcium indicator as described in Subheading 3.3.1.
3. Spot an aliquot (5–10 μL) of the indicator-loaded extract on a piece of Parafilm and place it on the microscope stage.

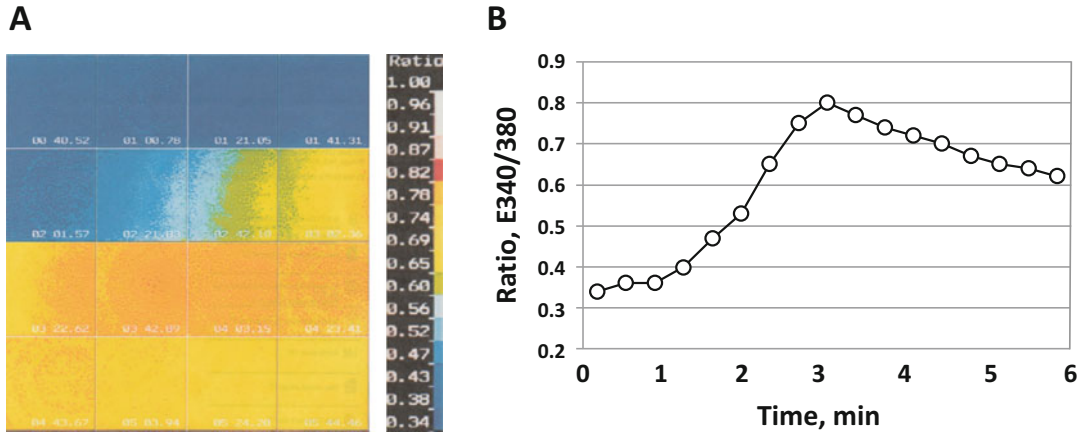

Fig. 4 Calcium wave in the extract of *Xenopus* eggs. The calcium signal was initiated in a 10 μL droplet of the low-centrifugation egg cytoplasmic extract by the addition of 50 nL of 10 μM IP3 solution. The extract contained ratiometric calcium indicator Fura-2 at 2 μM concentration. The field of microscopic observation covered a central part of the extract droplet. The activator solution was added on the right side of the microscopic field, and the calcium wave propagated leftwards. (**a**) Serial pictures of an extract area were taken at 20-s intervals, and the ratio of fluorescent signals at Ex 340/380 was visualized. The ratio color scale is shown in the right-hand vertical panel. (**b**) Cumulative kinetics of the calcium signal in a central area of the observation field

4. Monitor the fluorescent signal for several minutes to get a reference for the resting calcium level in the non-stimulated extract. The signal should remain stable.
5. Inject a small volume (10–50 nL) of the activator-containing water-based solution into the extract droplet with a microinjector. The equipment and needles, which are used for cytoplasmic collections from *Xenopus* oocytes and eggs, can also be used for microinjections (Fig. 3c).
6. Monitor and record propagation of the calcium wave in the extract droplet using a CCD camera-equipped fluorescent microscope.

Calcium release from the intracellular stores incorporated in the extract originates from the site of microinjection and spreads through the entire body of the extract droplet, as presented in Fig. 4.

3.5.2 Initiation of the Calcium Signal in Capillaries

Initiation of the calcium signal in the extract-filled glass capillaries is performed by uploading a small volume of the activator-containing solution into a capillary.

1. Monitor the fluorescent signal in a capillary filled with the indicator-uploaded extract for several minutes to get a reference for the resting calcium level in the non-stimulated extract. The signal should remain stable during observation.

2. Spot an aliquot (1–2 μL) of activator-containing solution on a piece of Parafilm.
3. Move the capillary tip into the droplet of activator solution under microscopic observation.
4. Withdraw a small volume (2–5 nL) of activator solution into the capillary (*see* **Note 18**).

The calcium signal originates at the capillary tip and propagates along the capillary, as presented in Fig. 5 (see the following section for details).

3.6 Detection and Quantification of the Calcium Signal

Continuous recording of fluorescence provides the most detailed information about the calcium signal. Also, the kinetics of calcium response in *Xenopus* oocyte and egg extracts presented in Figs. 4 and 5 suggest that extract imaging every 15–20 s is sufficient to reliably detect and characterize the calcium signal in the extracts. The onset of the calcium signal may show an initial lag period of 30–60 s, depending on the nature of signal inducer.

1. Record fluorescence of the UV-excitable ratiometric probe Fura-2 at Ex = 340 and 380 nm and Em = 510 nm using the IX83 imaging system or the high-frame digital CCD imaging ARGUS/HISCA system (*see* **Note 19**).
2. Analyze the ratio of signal intensities at the two recorded wavelengths (340 and 380 nm) with the built-in software. The emission ratio at the two wavelengths is calcium concentration dependent. It provides information about calcium levels in the extract. As an example, imaging of the calcium signal initiated in a droplet of CSF-arrested egg extract by addition of 10 μM IP3 in the presence of Fura-2 and signal kinetics is presented in Fig. 4.
3. To control upload of Fura-2 in a non-ratiometric mode, observe probe fluorescence with the near-UV filters (Ex = 330–385 nm and Em > 420 nm), as presented in Fig. 2.
4. When applying Fluo-8 (Ex/Em = 490/520 nm), detect fluorescence of the indicator with the optical interference filters optimized for detection of GFP. The CellSens Standard software (Olympus, Japan) can be used for calcium signal imaging and recording. Imaging of the calcium signal in a glass capillary filled with the Fluo-8-preloaded *Xenopus* oocyte extract is presented in Fig. 5.
5. Analyze the obtained fluorescent images with image processing software to further depict the calcium signal. The freeware ImageJ software of the National Institute of Health can be used to quantify and profile the signal [22]. The tool can be freely downloaded from https://imagej.nih.gov/ij/.

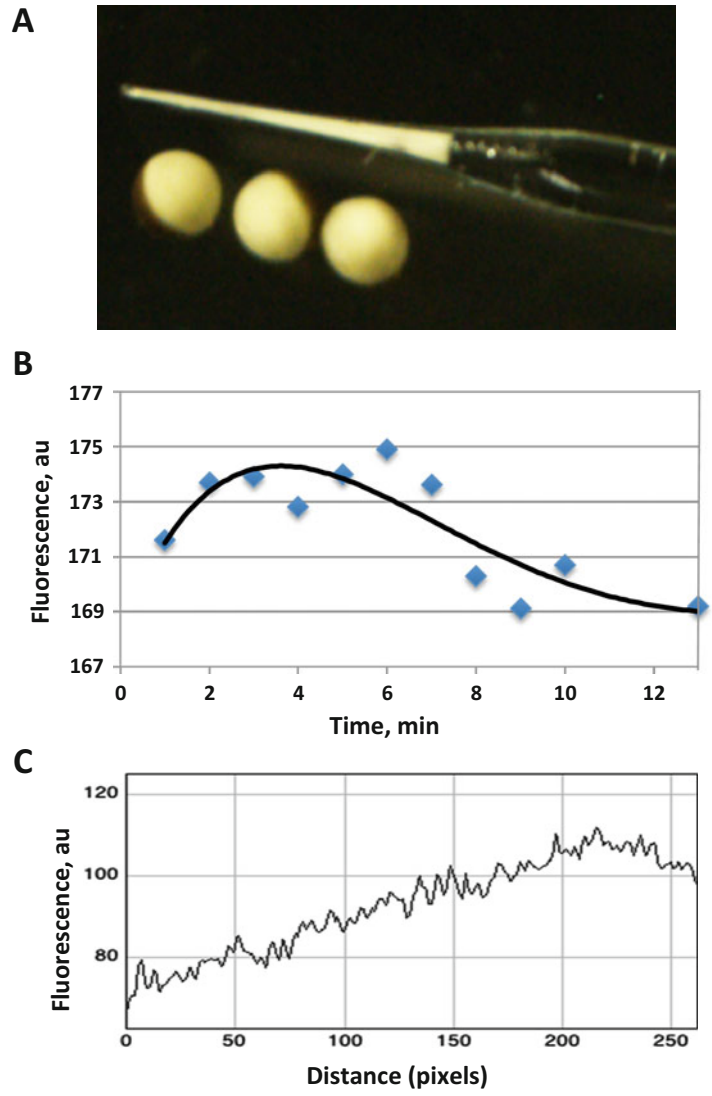

Fig. 5 Detection of the calcium signal in a glass capillary filled with *Xenopus* oocyte extract. The cell-permeable calcium indicator Fluo-8AM was uploaded in the oocyte, as detailed in the legend to Fig. 2. The calcium signal was initiated by uploading 10 μM IP3 solution at the capillary opening, as described in Subheading 3.5.2. (**a**) The tip of the extract-filled capillary observed using the SZX16 fluorescent microscope at 20× magnification. To indicate capillary size, three grown-up oocytes are shown in the panel. (**b**) Cumulative kinetics of the calcium signal in a central area of the capillary tip. (**c**) The profile of fluorescent signal along the whole length of capillary tip observed at 10 min after initiation of the signal

Alternatively, the Image Gauge software (FujiFilm, Japan) can be employed for image quantification. The lag period, speed of propagation, height, profile, and total area of the calcium signal should be evaluated.

4 Notes

1. *Xenopus* frogs attain sexual maturity within 10–12 months after their birth. Although the female frogs can deposit eggs up to four times a year, the best grown-up matured eggs can be obtained if the intervals between egg depositions exceed 6 months.
2. Calcium is included in the isolation media of oocytes and eggs to improve cell viability. However, residual free calcium in the cell-free extracts may dampen the detection of the specific calcium signal. Thus, calcium-free buffers are used later at the stage of extract preparation (*see* Subheading 3.2).
3. A plastic grid can also be used. In addition, other methods can be employed to immobilize oocytes and eggs during cytoplasmic collections and injections. Some researchers use indented Parafilm attached at the bottom of a Petri dish or a custom-made plastic injection sledge. It is also possible to hold oocytes still in place with forceps.
4. Fura-2 and Fura-2AM are the ratiometric fluorescent high-affinity (Kd~140 nM) calcium indicators. Fura-2 is not cell permeable and should be added directly to oocyte and egg extracts, whereas Fura-2AM can cross the cell membrane and may be pre-loaded. Fluo-8AM is a cell-permeable green fluorescent calcium indicator of medium affinity (Kd~390 nM). Its fluorescence increases upon calcium binding.
5. Specificity of PP2 toward Src family kinases is higher than that of PP1. PP3, an inactive analog of PP1 and PP2, should be used in control experiments to rule out nonspecific effects of the Src kinase inhibitors.
6. U-73343, an inactive analog of the PLCγ inhibitor U-73122, should be used in control experiments to rule out nonspecific effects of the inhibitor.
7. The exact duration of cysteine treatment depends on egg batch. The process of dejellying should be visually controlled and stopped when most eggs appear to lose jelly layer. Excessive incubation in the presence of cysteine leads to egg damage and lysis.
8. Almost complete removal of follicle cells is necessary to produce cell-free extracts by the protocol provided in this chapter, as the follicle layer prevents oocyte crushing by low-speed centrifugation. However, prolonged incubation with collagenase decreases oocyte quality, as it can be judged from irregular coloring of the animal hemisphere. The extent of defolliculation and oocyte quality can be checked under a dissecting microscope.

9. Cytochalasin B is added to the extract buffer and to cell-free oocyte/egg extracts to decrease viscosity of the extracts, which can otherwise polymerase, especially, when used in small volumes.
10. The extracts prepared from mature metaphase-arrested *Xenopus* eggs should retain high activity of CSF and MPF, indicating that calcium release in the eggs and extracts has not been initiated inadvertently. Inactivation of CSF and MPF is triggered by calcium-dependent degradation of cyclin B and Mos [7]. Adding EGTA in the XB buffer at this stage eliminates residual free calcium and calcium leaked from intracellular stores during extract preparation, thereby preventing calcium-dependent inactivation of CSF/MPF and meiotic exit before the experiment.
11. Due to differences in oocyte/egg quality and details of preparation process, the resulting extracts exhibit a great variation in quality and have low storage stability. The extracts of a single batch prepared on the same day should be used for comparative calcium signaling studies, including controls.
12. The total volume of added solution(s) should not exceed 10% of the extract volume. A greater dilution may significantly compromise calcium signaling in the extracts.
13. Although the higher concentrations of calcium indicators elicit the stronger fluorescence, they can dampen the specific calcium signal in the activated extracts.
14. Rigorous vortex mixing of the extracts should be avoided as it can affect structural integrity and spatial organization of the intracellular calcium stores.
15. A hydraulic, oil-filled microinjector is recommended because it provides a better sample control and can generate higher pressures than a pneumatic system.
16. To improve reproducibility of the results, cytoplasmic collections should be taken from the same area of the oocyte cytoplasm. It was demonstrated that the oocytes can withstand up to eight cytoplasmic collections and the loss of ~10% of their cytoplasm with little effect on their viability [21].
17. Mature, meiotically arrested *Xenopus* eggs can be easily activated by pricking, making unfeasible repeated cytoplasmic collections from these cells.
18. A larger volume of the activator-containing solution will dilute the extract in the capillary and interfere with initiation and propagation of the calcium signal (*see* also **Note 12**).
19. Detection of Fura-2 fluorescence at two excitation wavelengths allows more specific calcium detection without concern about dye concentration and absolute signal intensity. However, it is

also possible to use Fura-2 in a non-ratiometric mode, by measuring its overall UV-excited fluorescence, as demonstrated in Fig. 2.

Acknowledgments

This work was supported by the Collaboration Research grant 281027 from Kobe University to A.A.T. and the Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan 15K07083 to K-I.S.

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Chapter 5

Membrane Microdomains as Platform to Study Membrane-Associated Events During Oogenesis, Meiotic Maturation, and Fertilization in *Xenopus laevis*

Ken-Ichi Sato and Alexander A. Tokmakov

Abstract

Studies on the egg plasma membrane-associated tyrosine kinase Src have shed light on the identity of the molecular machinery that is responsible for gamete interaction and possibly fusion in African clawed frog *Xenopus laevis*. Here we describe our protocol for identifying and analyzing molecular and cellular machinery that contributes to a variety of biological processes in the course of oogenesis, oocyte maturation, egg fertilization, and early embryogenesis in *Xenopus*. Our current special interest is to evaluate the hypothesis that the oocyte/egg membrane microdomain (MD)-associated uroplakin III-Src system is responsible for mediating sperm-egg membrane interaction/fusion signal to the oocyte/egg cytoplasm to initiate embryonic and zygotic development in this species. Therefore, this chapter contains a brief introduction to biology of oocytes and eggs in *Xenopus* and addresses the following questions: (1) What is oocyte/egg MD? (2) Why do we study oocyte/egg MD? (3) How to manipulate oocyte/egg MD? (4) What has been achieved by oocyte/egg MD studies? (5) What are the next steps in oocyte/egg MD studies?

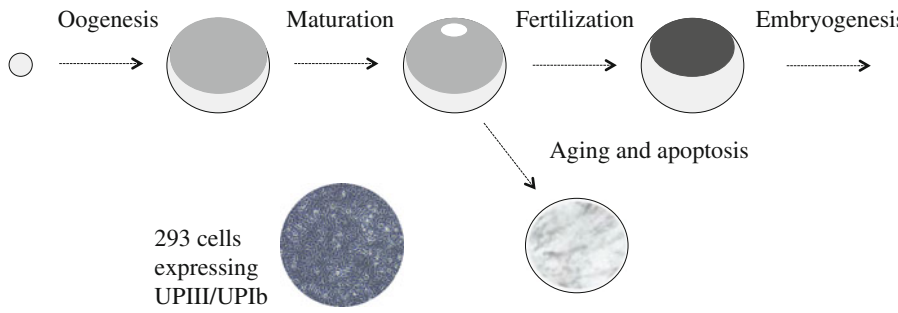
Key words African clawed frog, Signal transduction, Src, Tyrosine phosphorylation, Uroplakin III

1 Introduction: Biology of Oocyte and Egg in *Xenopus Laevis*

1.1 Oocyte at Germinal Vesicle (GV) Stage

Adult females of *Xenopus laevis* retain in their abdominal space a pair of ovarian fragment that usually holds a plenty amount of oocytes, whose oogenesis stage is very asynchronous: all stages of oocyte growth are found in the ovary at any time [1]. According to the classification by Dumont [2], the oocytes are designated from stage I through VI depending on their appearance and diameter (Fig. 1). During the progression of oocyte growth, which requires more than 8 months, the cells continue to increase in size by uptaking several substances from liver and by producing yolk materials inside the cells. In contrast, the cell cycle of growing oocytes is stably paused at prophase of the first meiosis.

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_5,



3.1 Preparation of MD from GV/MII oocytes, UF/F eggs, aged oocytes, and 293 cells

→ 3.2 In vitro fertilization
→ 3.3 Snap-shot identification of MD components
→ 3.4 In vitro reconstitution of fertilization signaling
→ 3.5 Generation of monoclonal antibody against MD/non-MD proteins
→ 3.6 Analysis of animal-vegetal polarity in MD

Fig. 1 Sequence of events associated with *Xenopus* oocyte and egg: oogenesis, maturation, fertilization, early embryogenesis, and apoptosis. Summary of the "Methods" section of this chapter, from the preparation of MD through a variety of its application, is also shown

1.2 Oocyte Maturation

Meiosis is the process by which diploid germ-line cell (i.e., oocyte and spermatocyte) reduces their genetic information by half to generate the haploid cell (i.e., egg and sperm). The haploid gametes fuse with each other to create a genetically new, diploid individual. Oocyte maturation, which is necessary for oocytes to acquire an ability to be fertilized, has been studied in many species of vertebrates and invertebrates; studies on frog systems in particular have contributed to a detailed understanding of its biochemical nature. In almost all vertebrates, the oocyte meiotic cell cycle starts during the fetal stage, and then it is arrested in the first meiotic prophase (Pro-I), which lasts for several months or years in the follicular or ovarian microenvironment. The duration of the arrest depends on the species. Physiological or experimental hormonal action on oocytes stimulates resumption of the cell cycle and further progression of the meiotic cell cycle is paused again, in many but not all species, at the stage of second meiotic metaphase II (MII) (Fig. 1). Different kinds of molecules, for example, extracellular signaling molecules (e.g., progesterone) and oocyte surface receptors (e.g., receptor for progesterone), and intracellular signaling molecules (e.g., maturation-promoting factor, mitogen-activated protein kinase) are involved in these cell cycle events. The cytoplasmic events associated with oocyte maturation have been studied extensively; however, the knowledge about the membrane-associated events in oocyte maturation is still quite limited.

1.3 Fertilization and Activation of Development

Sperm-egg interaction and fusion, that is, fertilization, promote the exit of the oocyte from MII arrest (Fig. 1). Sperm-induced resumption of MII arrest and initiation of zygotic development is called "egg activation," which is characterized by many biochemical and cell biological reactions, for example, Ca^{2+} oscillations, cortical granules exocytosis, block to polyspermy, extrusion of polar body, formation of male and female pronuclei and their fusion, recruitment of maternal mRNAs, and initiation of DNA synthesis for mitotic divisions to unveil the complete developmental program. The wave of Ca^{2+} initiates at the site of sperm binding/fusion and it is followed by a wave of intracellular Ca^{2+} traversing the entire cytoplasm of the egg. Sperm-mediated Ca^{2+} oscillations or Ca^{2+} release with wavelike propagation within the fertilized eggs activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Activated CaMKII and polo-like kinase simultaneously phosphorylate and inactivate Emi2. Cdc20, an activator of anaphase-promoting complex/cyclosome (APC/C) that is maintained to be inactive by Emi2 during MII arrest, is released from the inactivated Emi2 and binds to APC/C, resulting in the forming of an active APC/C complex. Activated APC/C induces the degradation of cyclin B, an activating partner of cdc2 kinase, by which MPF activity declines. MII arrest also involves the progesterone-induced translation of Mos protein kinase, and its downstream protein kinase cascade involving MAPK kinase, MAPK, and ribosomal S6 kinase (RSK). Active RSK induces spindle assembly checkpoint (SAC) protein activation and thereby inhibits APC/C to maintain MII arrest. At fertilization, Mos undergoes degradation, by which the downstream kinase cascade is soon inactivated. At the end of this process, sister chromatids are segregated, the second polar body is extruded, and the first embryonic cell division starts.

As in the case of oocyte maturation, sperm-induced cytoplasmic events have been extensively studied so far. However, plasma membrane-associated events and its molecular details are not yet understood.

1.4 Aging and Apoptosis of Postovulatory and Unfertilized Egg

The postovulatory oocyte, which undergoes progesterone-induced maturation and secondary arrest at MII, mimics the action of egg activation due to aging, increases cytoplasmic Ca^{2+}, and induces exit from the MII arrest [3–5]. However, it does not progress further and undergoes arrest again in a new metaphase-like stage called MIII in a few vertebrate species. The mechanism for MIII arrest remains poorly understood. In aged eggs, insufficient Ca^{2+} release and sufficient CSF activity are still present to stabilize the residual or newly formed MPF activity, resulting in MIII arrest. Some recent reports have demonstrated that the unfertilized aged eggs undergo apoptotic processes, such as decoloring and swelling of the cell, inactivation of MAPK, and transient activation of caspase 3/7 (Figs. 1 and 2). The physiological importance of postovulatory

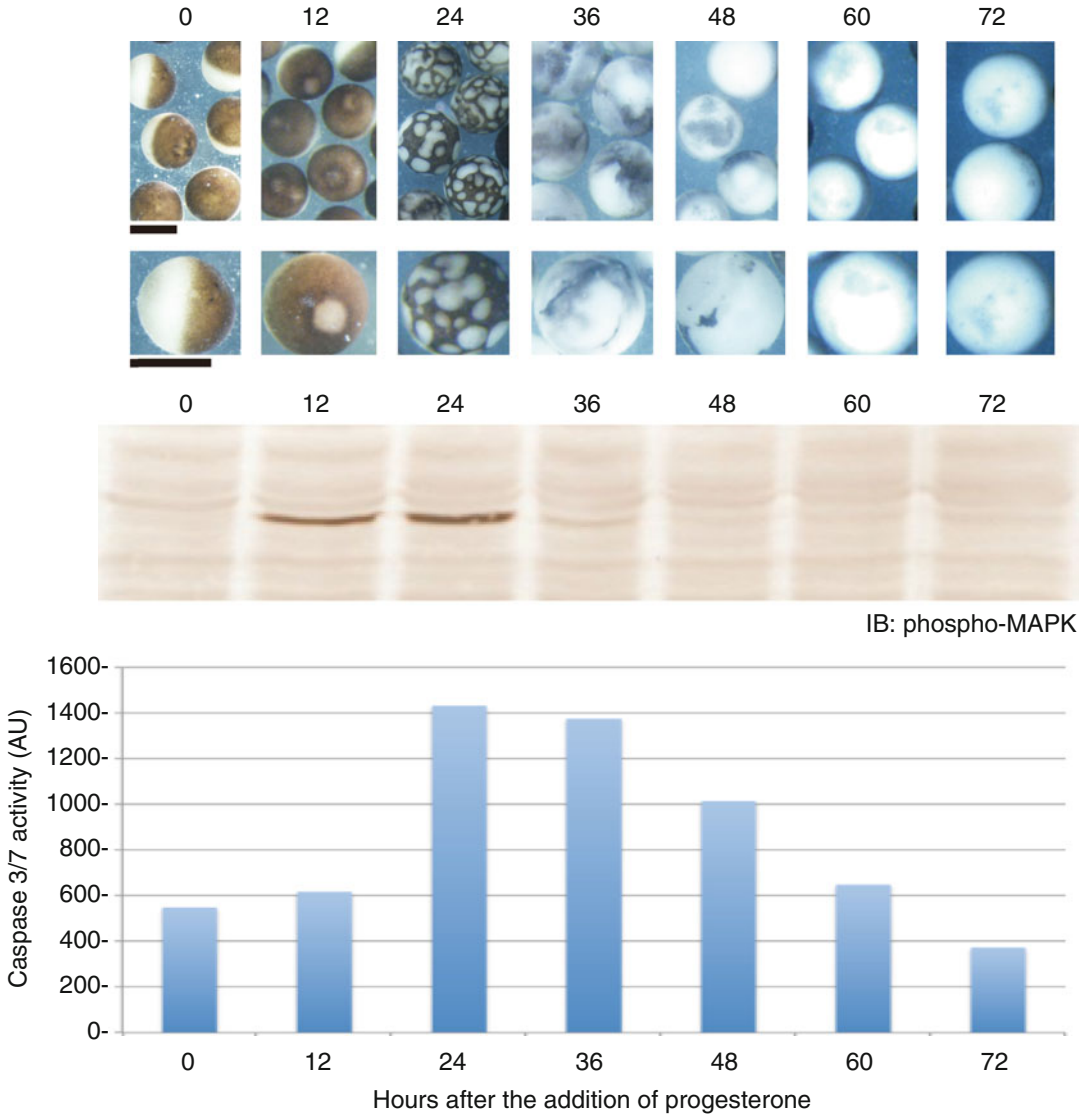


Fig. 2 Progesterone-induced maturation, aging, and apoptosis of oocytes in *Xenopus laevis*. Upper panels: Morphology of oocytes in the course of progesterone treatments (0–72 h). Middle panel: Phosphorylation of MAPK. *IB* immunoblotting. Bottom panel: Activity of caspase 3/7. *AU* arbitrary unit

apoptosis of oocyte/egg in *Xenopus laevis*, a species of external fertilization, has recently been postulated and discussed. Plasma membrane-associated events in the course of aging and apoptosis in oocyte and egg provide new problems awaiting detailed investigation.

1.5 What Is MD? Why Study MD?

According to Laude and Prior [6], "the plasma membrane consists of a mosaic of functional microdomains facilitating a variety of physiological processes associated with the cell surface."

Experimentally, the membrane microdomain or MD is prepared as low-density, detergent-insoluble membrane (LD-DIM) that is enriched in membrane lipid components such as cholesterol and sphingolipids, and a specific subset of proteins. The rationale to study oocyte/egg MD is that pharmacological and biochemical experiments have demonstrated that MD of unfertilized *Xenopus* egg seems to act as a platform for sperm-induced Src tyrosine kinase signaling at fertilization.

1.6 UPIII-Src System, a Mediator of Sperm-Egg Interaction and Egg Activation

It was 1996 when we reported that the egg-associated tyrosine kinase Src (called p57 kinase or *Xenopus* tyrosine kinase) is activated within minutes of in vitro fertilization of *Xenopus laevis* [7]. The activated Src has been shown to contribute to phosphorylation and activation of phospholipase Cγ, by which it also contributes to inositol 1,4,5-trisphosphate-dependent intracellular Ca^{2+} release within the fertilized eggs. Src family tyrosine kinases (SFKs) play important roles in sperm-induced Ca^{2+} response in several species, sea urchin, starfish, rat, ascidian, mouse, and frog eggs [8]. Upstream interaction partner for Src has also been identified from comparative phosphorylation studies of unfertilized and fertilized *Xenopus* eggs. Uroplakin III (UPIII), a 30 kDa and glycosylated single-transmembrane protein, was initially identified as a predominantly tyrosine-phosphorylated protein that localizes to LD-DIM fractions of fertilized *Xenopus* eggs. Further study of UPIII has shown that it has sperm-interacting function as a target of sperm protease. *Xenopus* fertilization requires tryptic protease activity of sperm at the level of gamete plasma membrane interaction. Its pharmacological inhibition results in a failure of sperm-induced activation of Src and embryonic development. UPIII has a binding partner, uroplakin Ib, a tetraspanin transmembrane UP family protein that contributes to UPIII's exit from the endoplasmic reticulum and membrane localization. The UPIII-UPIb complex on the egg surface physically associates with the ganglioside GM1, a well-known component of MD. Matrix metalloproteinase-2 (MMP-2), a sperm component involved in egg membrane interaction, was shown to directly bind to GM1 in vitro [9]. The ganglioside GM1 has a negative charge in its molecular structure; on the other hand, the HPX domain of sperm MMP-2 has a positive charge in its structure, suggesting that such an electrically complementary nature of these molecules could serve as a basis for their interaction, and that this interaction could provide the means for a voltage-dependent gamete interaction at the plasma membrane level [9]. The mechanism by which sperm-induced proteolysis of UPIII induces activation of the egg cytoplasmic Src remains unclear. More recent study has demonstrated that the UPIII-Src system in egg MD acts not only for receiving and transmitting egg activation signals from fertilizing sperm, but also for "activating" the fertilizing sperm through their membrane interaction [10].

2 Materials

1. A23187 (Sigma): Stock at 10 mM in dimethyl sulfoxide (DMSO); store at −80 °C.
2. 4-Amidinobenzylsulfonyl fluoride hydrochloride (APMSF) (Wako): Stock at 100 mM in distilled water; store at −30 °C.
3. ATP: Stock at 100 mM, 1 mM DTT, in distilled water; store at −30 °C.
4. Animals: *Xenopus* adults were obtained from a breeding facility (e.g., Shimizu Laboratory Supplies (Kyoto Japan)). The animals were maintained in dechloride tap water (1.2–1.5 L/animal) at 18–22 °C and fed artificial, floating feed twice a week.
5. Antibodies: Rabbit polyclonal antibodies against a C-terminal sequence of UPIII (UPIII-CT) and an internal sequence in the non-catalytic domain of Src1/2, and a rabbit antibody against the extracellular domain of UPIII (GST-UPIII-ED) were prepared as described previously [11, 12]. Anti-phosphotyrosine antibody PY99 and a phospho-specific anti-active Src family kinase antibody pY418 were from Santa Cruz Biotechnology and Biosource International, respectively. Polyclonal antibodies against mitogen-activated protein kinase (MAPK) and its active form (pMAPK; 9101L) were purchased from Cell Signaling Technology. A monoclonal antibody against biotin (clone BN-34) was obtained from Sigma-Aldrich. A monoclonal antibody against mammalian PLCγ was purchased from Upstate Biotechnology. Anti-FLAG antibody (M2 clone) was from Sigma.
6. Cathepsin B from bovine spleen: Stock at 5 mg/mL in distilled water; store at −30 °C.
7. Chorionic gonadotropin from human (hCG): Stock at 2500 International Units/mL in solvent provided by the supplier; store at 4 °C.
8. Cultured cells of mammalian species: Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Subconfluent cells were used for transfection experiments.
9. Cytostatic factor-arrested (MII-arrested) egg extracts (CSF extracts): Detail of the preparation has already been described [13].
10. 1× DeBoer's solution (DB): 110 mM NaCl, 1.3 mM KCl, and 0.44 mM $CaCl_2$ adjusted to pH 7.2 with $NaHCO_3$; its concentrated stock solution is 20× DB; store at 4 °C.
11. Dejellying solution: DB containing 2% (w/v) cysteine, adjusted to pH 7.8 with NaOH.

12. Dithiothreitol (DTT): Stock at 1 M in distilled water; store at −30 °C.
13. Expression vectors: Complementary DNA (cDNA) constructs for expression of UPIII and FLAG-tagged *Xenopus* UPIb were constructed using p3×FLAG-CMV-14 vector and pCMV-tag5A, respectively, as described previously [11, 12, 14]. Preparation of p3×FLAG-CMV-14 containing wild-type Src (Src-WT) was as described [15]. For expression of mutant UPIII (UPIII-RR/AA), in which the Arg187-Arg188 motif in the conserved juxta-transmembrane sequence was substituted by the Ala187-Ala188 mutations, the pCMV-tag5A/UPIII vector was subjected to polymerase chain reactions (PCR) with mutagenic primers (5′–3′, underlined are mutated nucleotides): forward, ACATGGCCTGGCGCAGCGAGTGGTGGGATG; reverse, CATCCCACCACTCGCTGCGCCAGGCCATGT. HEK293 cells: culture in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) bovine serum albumin at 37 °C/5% (v/v) CO_2 humidified atmosphere [14].
14. Extraction buffer A: 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM ethylenediaminetetraacetic acid (EGTA), 10 mM β-mercaptoethanol; 10× stock, store at 4 °C.
15. Extraction buffer B: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM EGTA, 50 mM sucrose, and 50 mM HEPES-NaOH titrated to pH 7.8.
16. Ficoll: Molecular weight 400,000.
17. Guanine nucleotide GTPγS: Stock at 10 mM in distilled water, store at −80 °C.
18. Guanine nucleotide GDPβS: Stock at 10 mM in distilled water, store at −80 °C.
19. Immunoblotting instrument: Trans Blot Semi-dry Transfer Cell (Bio-Rad).
20. Isoelectrofocusing (IEF) system: Ettan IPGphor (GE Healthcare) and Immobiline dry strips of pH range 3–10 or 4–7 were used according to the manufacturer's instruction.
21. 0.33× Modified Ringer's solution: 33.3 mM NaCl, 0.6 mM KCl, 0.33 mM $MgCl_2$, 0.66 mM $CaCl_2$, and 1.67 mM HEPES-NaOH, pH 7.8.
22. 5× Laemmli's sodium dodecyl sulfate (SDS) sample buffer: 200 mM Tris phosphate, pH 6.7, 5% SDS, 100 mM DTT, 50% (w/v) sucrose, 0.025% (w/v) bromophenol blue.
23. Leupeptin: Stock at 100 mg/mL in distilled water; store at −30 °C.

24. LY294002, an inhibitor for phosphatidylinositol 3-kinase: Stock at 5 mM in DMSO, store at −80 °C.
25. Messenger RNAs (mRNAs): mRNAs encoding *Xenopus laevis* UPIb, UPIII, and Src (*Xenopus* Src2) were purified from *Xenopus* liver using the Quick Prep Micro mRNA Purification Kit (GE Healthcare).
26. Methyl-β-cyclodextrin (MβCD) was purchased from Wako Pure Chemicals.
27. Phospholipase C (PLC) inhibitor U-73122 and its inactive analog U-73343: Stock at 5 mM in DMSO, store at −80 °C.
28. Pregnant mare serum gonadotropin (PMSG): Stock at 2500 International Units/mL in distilled water, store at −30 °C.
29. Progesterone (PG), a water-soluble derivative: Stock at 10 mM in distilled water, store at 4 °C.
30. Protein-tyrosine kinase (PTK) inhibitor PP2 and its inactive analog PP3, genistein, and its inactive analog daidzein: 5 mM stock in DMSO; store at −80 °C.
31. Serum albumin from bovine (BSA): Fraction V, crystalline grade.
32. Na_3VO_4 (Wako, Osaka, Japan): Stock at 500 mM in distilled water; store at 4 °C.
33. SW28 rotor: Beckman, Palo Alto, CA.
34. Staurosporine: Stock at 1 mM in DMSO, store at −30 °C.
35. 0.5× Steinberg solution (SB): 29 mM NaCl, 0.34 mM KCl, 0.17 mM $CaCl_2$, 0.43 mM Mg_2SO_4, and 2.3 mM Tris–HCl, pH 7.4.
36. Sucrose buffer: Stock at 85% (w/v) solution in extraction buffer A; store at 4 °C.
37. Synthetic peptides that correspond to residues 177-191 of UPIII (GRR peptide, SSGTIDTWPGRRSGG) and a mutated version (GAA peptide, SSGTIDTWPGAASGG, in which di-arginine residues are mutated to alanine residues): Stock at 5 mM in distilled water or 0.9% (w/v) sodium chloride, store at −30 °C.
38. Transfection reagent Effectene (Qiagen, Germany): Store and use at room temperature according to the manufacturer's instruction.
39. Triton X-100 (Sigma): Stock at 25% (v/v) in distilled water; store at 4 °C.
40. Proteases such as lysyl-endopeptidase (*Achromobacter lyticus* protease I) and Asp-N endoprotease: Stock at 1–5 mg/mL in distilled water, store at 4 °C.

41. Ovarian immature oocytes at GV stage (meiotic stage of Pro-I) were prepared from *Xenopus* adults primed with PMSG and/or hCG. Mature oocytes at meiotic stage of MII were prepared by the addition of progesterone as described previously ([7, 16]; Tokmakov et al. 2005). Sperm for in vitro fertilization and in vitro reconstitution of signaling events were prepared from *Xenopus* adults primed with hCG as described previously [10, 17].

3 Methods

3.1 Membrane Microdomains (MD)

Conventional (normal scale) procedures for preparation of MD from oocyte and egg of 700–800 or more cells per sample are outlined below.

1. Oocyte or egg samples are mixed with fivefold volume of ice-cold extraction buffer A supplemented with 1 mM Na_3VO_4, 10 μg/mL leupeptin, 20 μM APMSF, 150 mM NaCl, and 250 mM sucrose and homogenized with a 7 mL Dounce tissue grinder.
2. The homogenates are removed of debris and yolk materials by centrifugation at 500 × *g* for 10 min, and the supernatants are centrifuged at 150,000 × *g* for 20 min.
3. The fluffy layer of the pellet (crude plasma membranes) is carefully collected and mixed with 25% (v/v) Triton X-100 to yield a final concentration of 1% (v/v) Triton X-100.
4. The mixtures are homogenized again with the Dounce tissue grinder, incubated on ice for 10 min, and mixed with equal volumes of ice-cold extraction buffer A containing 150 mM NaCl and 85% (w/v) sucrose (sucrose buffer).
5. The resulting mixtures (5 mL) are layered first with 19 mL 30% sucrose and second with 12 mL 5% (w/v) sucrose in the same buffer.
6. The samples are centrifuged at 144,000 × *g* for 24 h in an SW28 rotor.
7. After the centrifugation, 3 mL aliquots of 12 fractions are collected from the top to the bottom of the tubes (*see* **Notes 1–3**).

3.2 In Vitro Fertilization

In vitro fertilization of ovulated and unfertilized eggs is performed as described [17, 18].

1. Egg-jelly water is prepared by incubation of 3 volumes of unfertilized eggs with 8 volumes of DB, for 10 min at room temperature.

2. Sperm are pretreated with the egg-jelly water for 2–5 min at room temperature, by which sperm achieve active motility and undergo the acrosome reaction effectively and the success rate of egg activation is increased.
3. Freshly ovulated, unfertilized eggs are prepared on a plastic dish.
4. Eggs are pretreated with a rabbit antibody against GST-UPIII-ED at the concentrations of 0.1–10 μg/mL, for 15–25 min at room temperature.
5. Eggs are washed several times with buffer solution.
6. Eggs are then treated with the egg-jelly water-treated sperm (10^6 sperm/mL).
7. Sperm and/or eggs are sometimes preincubated with MDs at the specified concentrations (*see* Subheading 3.4), and subjected to in vitro fertilization.

3.3 "Snapshot" Identification of MD Components

1. Aliquots of MD and non-MD fractions, usually 12 samples, that are prepared from GV oocytes, MII oocytes, spawned and unfertilized eggs, and fertilized eggs (each sample of 800–1000 cells), are separated by SDS-PAGE on 10% polyacrylamide gels.
2. For direct visualization of proteins, the gels are analyzed by protein stain with silver or Coomassie Brilliant Blue.
3. For detection of proteins or their posttranslational modifications and processing (e.g., tyrosine phosphorylation, proteolysis), the gels are subjected to immunoblotting with the specific antibodies, the target of which are either phosphotyrosine, Src, uroplakin III, or others.
4. Under this condition, ca. five oocyte/egg-equivalent amounts of proteins per fraction will be analyzed (*see* **Note 4**).

3.4 In Vitro Reconstitution of Signaling Events

To assess signaling events associated with sperm-egg interaction at fertilization, proteolysis and tyrosine phosphorylation of MD-associated UPIII and Src are reconstituted in vitro.

1. MDs prepared from either GV stage VI oocytes, progesterone-treated MII oocytes, unfertilized eggs (2.5–10 μg protein, equivalent to 30–60 oocytes or eggs), or HEK293 cells (3.3–10 μg protein, equivalent to 1.5×10^6 cells) are treated in the absence or presence of either of the following egg activators: 10^6/mL sperm, 5 U/mL cathepsin B, 1 mM GTPγS, 1 mM cAMP, 1 mM RGDS peptide, or 1 mM $CaCl_2$ at a final volume of 12.5–25 μL for 10 min at 30 °C for 10 min at 21 °C.
2. In some experiments, the mixtures of MDs and egg activators are co-incubated with one of the following: anti-UPIII IgG

(5 μg/mL), PP2 (5 μM), GDPβS (20 μM), LY294002 (100 μM), or MβCD (25 mM).

3. After incubation, the mixtures are supplemented with 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 1 mM ATP.
4. The mixtures are further incubated at 21 °C for 10 min.
5. The reactions are terminated by the addition of 50 μL of Laemmli SDS sample buffer [19].
6. The protein samples are separated by SDS-PAGE on 10% (w/v) gels.
7. The SDS gels are subjected to immunoblotting with the use of antibodies of interest.
8. Signaling events such as tyrosine phosphorylation of xSrc and UPIII and partial proteolysis of UPIII are evaluated.
9. Dephosphorylation of MAPK is also reconstituted in vitro using MDs and cytostatic factor-arrested egg extracts (CSF extracts) according to methods described previously [13, 20, 21].

For further insights obtained so far by this reconstitution system, *see* **Notes 5**–**7**.

3.5 Generation of Monoclonal Antibodies

1. Crude plasma membranes of unfertilized eggs, which are obtained after the first round of ultracentrifugation in the preparation of MD fractions (*see* Subheading 3.1), are employed as an immunogen to generate rat monoclonal antibody against the MD/non-MD-associated proteins.
2. Rat is used for immunizing animals because its probability to produce immunoglobulin other than IgM is thought to be higher than other animals such as mouse.
3. First screening of immunoreactivity of the rat hybridoma clones is performed by enzyme-linked immunosorbent assay and by immunoblotting of the Triton X-100-solubilized crude plasma membranes.
4. Positive clones are selected and further evaluated by immunoblotting of the MD and non-MD fractions that are prepared from unfertilized eggs and by indirect immunofluorescent staining of the dejellied unfertilized eggs (*see* **Note 8**).

3.6 Animal-Vegetal Polarity

Polarity of plasma membranes in oocyte has been one of the classical issues in biology of *Xenopus* oocyte that are yet under investigation [22].

1. To assess animal-vegetal polarity of the oocyte and egg, MD fractions are prepared from the fragments of oocyte/egg that have been prepared by manual cutting of the cells into halves at the marginal line of the animal and vegetal hemispheres.

2. After the conventional separation and concentration of MD from crude plasma membranes by discontinuous sucrose density ultracentrifugation (*see* Subheading 3.1), the animal hemisphere-derived and vegetal hemisphere-derived MD samples are subjected to two-dimensional gel electrophoresis that involves IEF (first dimension) and SDS-PAGE (second dimension).
3. The SDS-PAGE gels are stained with silver and further analyzed for the protein identity by mass spectrometry [13, 23, 24] (*see* **Note 9**).

4 Notes

1. Fractions 3–6 are combined and stored as MD fractions (LD-DIMs: low-density, detergent-insoluble membranes), whereas fractions 10–12 are combined and stored as detergent-soluble non-MD fractions.
2. Alternative, small-scaled procedures for preparation of MD from oocyte or egg of 100–200 cells per sample or cultured cells of ~10^7 per sample are outlined below.
   (a) Triton X-100-solubilized cell extract (400 μL), which had been prepared from two cell dishes of 100 mm diameter, is brought to 42.5% (w/v) sucrose by mixing with equal volume of ice-cold buffer A containing 85% sucrose.
   (b) The resulting mixture (about 0.8 mL) is layered with 2.6 mL 35% (first) and 1.6 mL 5% sucrose (second) in buffer A.
   (c) The samples are centrifuged at 200,000 × *g* for 20 h in an SW55 rotator (Beckman).
   (d) After the centrifugation, 0.425 mL aliquots of 12 fractions are collected from the top to the bottom of the tube.
3. In some experiments, MD fractions, as prepared in Subheading 3.1, **steps 1–7**, are further processed as follows:

   Additional step (*AS*) 1: Combine and dialyze against the buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, and 10 mM β-mercaptoethanol with a dialysis membrane (molecular weight of 3500).

   AS i: The dialysis buffer is changed every 2 h three times.

   AS ii: The dialyzed fractions are centrifuged at 300,000 × *g* for 1 h.

   AS iii: The pellet is resuspended in 1.5 mL of buffer A and ultrasonicated for 1 min with an ultrasonic cell/tissue disrupter.

AS iv: The suspension solution is centrifuged with 1800 × *g* for 5 min to remove the debris.

AS v: The supernatant is collected as "concentrated membrane microdomains" and used for further manipulations.

4. This approach of "snapshot" experiment has allowed us to identify tyrosine-phosphorylated proteins in fertilized eggs, such as Src, PLCγ, and UPIII [25]. Alternatively, the combined MD (e.g., fractions 3–6) are subjected to two-dimensional electrophoresis that involved isoelectrofocusing using Ettan IPGphor IEF system as a first dimension and SDS-PAGE on 10% gels as a second dimension. In this experiment, oocyte/egg-equivalent amounts of 200–1000 cells can be analyzed in one gel. This alternative set of "snapshot" experiment enables us to visualize and record a kind of well-compartmentalized fingerprint of MD-associated proteins in oocytes and eggs (*see* also Subheading 3.6).
5. More recent study has demonstrated that the UPIII-Src system in egg MD acts not only for receiving and transmitting egg activation signals from fertilizing sperm, but also for "activating" the fertilizing sperm through their membrane interaction. Experiments using isolated MD in unfertilized eggs have shown that the MD fraction is capable of interacting with fertilizing sperm. This interaction enables sperm to fertilize eggs that are pretreated with an antibody that binds to the extracellular domain of UPIII. The antibody has been shown to inhibit sperm-induced proteolysis of UPIII and subsequent Src-dependent egg activation events. The results suggest that the exogenously added MD fraction "activates" sperm; otherwise, fertilization of the antibody-treated eggs is not possible. Such activation mechanism of the MD-treated sperm involves proteolysis of UPIII in the MD fraction and sperm protein kinase activity, the latter of which has been suggested by pharmacological inhibition studies. More detailed study to explore the membrane interaction between MD and sperm, for example, possible ligand function of the partially proteolyzed UPIII extracellular domain and the molecular identity of sperm protein kinase that is involved in the MD-induced "activation" of sperm, is awaited.
6. Another line of recent studies has demonstrated that the UPIII-Src system acquires its functional competency, that is, the ability to receive the sperm signal and to transmit it to the egg cytoplasm, during hormone-induced oocyte maturation. Immunochemical and surface biotinylation studies have shown that UPIII is expressed in ovarian immature oocytes from the beginning of oogenesis. Indirect immunofluorescent studies

have shown, however, that the UPIII extracellular domain becomes more accessible to the anti-UPIII extracellular domain antibody (as mentioned above) after hormonal treatment of oocytes for maturation. In vitro reconstitution experiments using isolated MD fractions of sperm-induced UPIII proteolysis and Src activation have also demonstrated that the responsiveness of MD of fully grown immature oocytes to sperm is much weaker than that of in vitro-maturing oocytes or of ovulated unfertilized eggs. The aforementioned sperm "activating" property is also weak in MD of immature oocytes. Taken these findings together, the MD-associated UPIII-Src system in MD seems to represent the first example of molecular machinery that undergoes maturation at the oocyte/egg plasma membrane level in *Xenopus laevis*.

7. These approaches for "in vitro reconstitution" have allowed us to demonstrate that MD signaling events associated with fertilization involve the UPIII-xSrc system that could be at least in part reconstituted with the use of sperm and isolated MD fractions.
8. As a result, we obtained three monoclonal antibodies. Interestingly, the target proteins of each monoclonal antibody (denoted as immunogens A, B, and C) are shown to localize to MD fraction alone (immunogen A), non-MD fraction alone (immunogen B), or both fractions (immunogen C). To our knowledge, immunogens A and B represent the first examples as either MD-specific or non-MD-specific oocyte/egg. Immunogens. Further molecular identification and functional analysis of the monoclonal antibody's target molecules are now under investigation.
9. The results obtained so far demonstrate that animal- and vegetal-hemisphere-associated MD contain proteins peculiar to either animal or vegetal hemispheres as well as those that are commonly present in both hemispheres.

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Chapter 6

Embryological and Genetic Manipulation of Chick Development

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Abstract

The ability to combine embryological manipulations with gene function analysis in an amniote embryo makes the chick a valuable system for the vertebrate developmental biologist. This chapter describes methods for those unfamiliar with the chick system wishing to initiate experiments in their lab. After outlining methods to prepare chick embryos, protocols are provided for introducing beads or cells expressing secreted factors, and for culturing tissue explants as a means of assessing development in vitro. Approaches to achieve gain of function and loss of function (morpholino oligonucleotides) in chick are outlined, and methods for introducing these reagents by electroporation are detailed.

Key words Chick, Embryo culture, Electroporation, Morpholino oligonucleotide

1 Introduction

Chick embryos have captivated developmental biologists since the days of Aristotle [1]. Large and externally developing, chick embryos are amenable to in vivo and in vitro manipulations that assay fate, inductive signaling interactions, and, more recently, gene function. Long a mainstay of limb, somite, neural crest, and spinal cord developmental studies, the ability to combine chick embryology with gain- or loss-of-function approaches has greatly increased the utility and popularity of the chick system. In addition to enabling a wider array of developmental inquiry in the chick, this new era makes possible comparative studies between the chick and other vertebrate model organisms. For example, when used in conjunction with a genetic system such as the mouse (particularly useful as both are amniotes and thus evolutionarily close), new experiments become feasible: the conservation of gene function can be evaluated, and chick embryological manipulations that are

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_6,

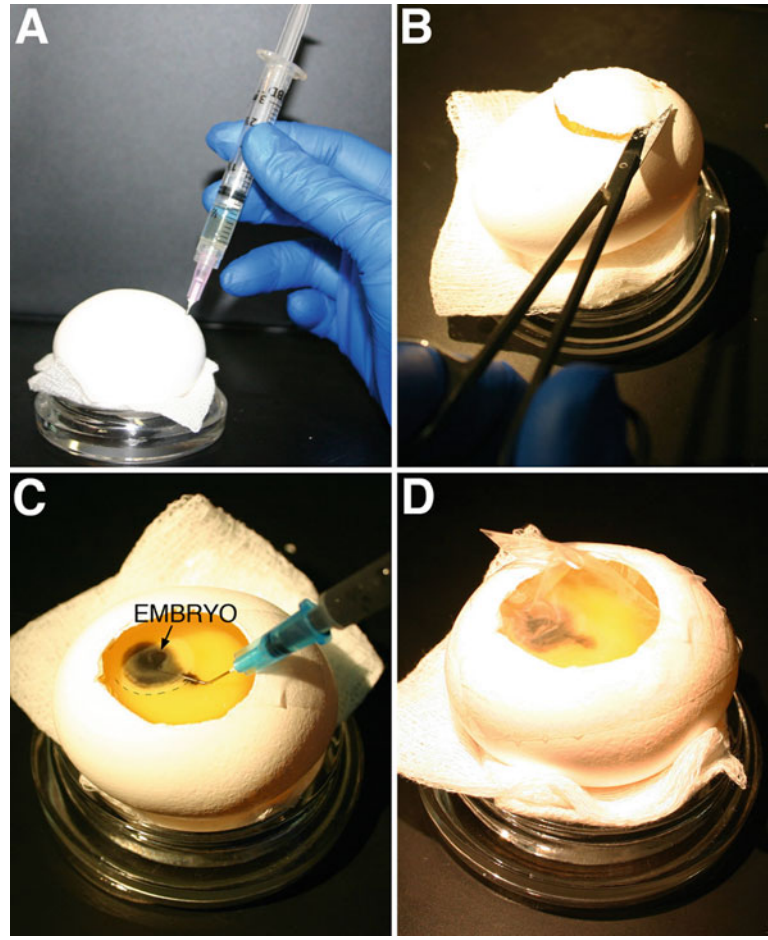

Fig. 1 Opening chicken eggs. (**a**) A 3 mL syringe outfitted with an 18 gauge needle is carefully inserted into the top side of the blunt end of the egg. The needle tip is introduced along the curvature of the shell to avoid puncturing the yolk, and 3 mL of albumin is withdrawn from the bottom of the egg. (**b**) One or two pieces of scotch tape are laid across the top of the egg, and a hole of 1.5–2 cm diameter is cut in the shell. (**c**) India ink diluted in Ringer's saline is injected under the embryo using a 1 mL syringe outfitted with a 25 gauge needle bent at a 45° angle, to enhance contrast. The needle tip is inserted just outside the perimeter of the lighter colored area opaca of the blastoderm (indicated with a dotted line along its lower edge in the photograph) and brought up under the embryo, where ink is expelled. (**d**) Following manipulation, eggs are sealed well with packing tape and placed back into 38 °C/100 °F incubator

impossible in the mouse can be used to further investigate mechanisms suggested by genetic analysis. As a result, a more complete and comprehensive view of a developmental process unfolds. This chapter serves as an introduction to chick methods. Basic protocols for culturing embryos and manipulating signaling or gene expression are described.

1.1 Culturing Chicken Embryos and Explants

A chicken embryo comes ready to use in an incubation vessel complete with its own culture medium. As a result, embryos can be manipulated in ovo (in the eggshell) by opening an access hole (Fig. 1). After procedures are completed, this hole can be sealed with packing tape to maintain a humid, sterile environment, and the embryo can be incubated to the desired stage in order to assay the consequences of any manipulations. Alternatively, if greater accessibility is needed, embryos can be cut from the yolk while attached to a filter paper frame (to maintain proper tension across the blastoderm) and cultured ex ovo (i.e., outside the eggshell, in vitro) up to 24 h on a substrate of albumin mixed with agar [2]. Working ex ovo can facilitate microsurgery, bead implantation, and electroporation of gain- and loss-of-function reagents, depending upon the stage and embryonic region being assayed. Finally, chick embryonic tissues may be dissected from the embryo and cultured in a three-dimensional collagen matrix (as in [3, 4]). The collagen supports the tissue so that it retains its normal conformation, in contrast to culturing tissue directly on a culture dish [5], which causes tissues to spread and adopt an artificial two-dimensional arrangement. With explant cultures, issues of commitment and cell-cell signaling can be addressed.

1.2 Manipulating Cell-Cell Signaling

As a model organism, chicken embryos are particularly useful to investigate the developmental consequences of secreted signals. Rather than bathing the entire embryo in a signal, cell pellets that produce the factor of interest (as in Ref. [6]) or beads soaked in purified protein (as in Ref. [7]) can be implanted to create a localized source of the signal that is gradually released and continuously replenished. The location of implanted cells or beads can be identified after a period of incubation, and neighboring cells assayed for changes in gene expression, altered morphology, or diverted migration.

1.3 Knocking Down Gene Function

Although chick is not amenable to classical genetic investigation, chick biologists can achieve transient knockdown of endogenous protein by RNA interference (RNAi; [8, 9]) or antisense morpholino oligonucleotides. Moreover, protocols using CRISPR/Cas9 to create genetic mutations recently have been developed [10–12]. This chapter covers the use of morpholinos.

Morpholino oligonucleotides (MOs) are modified nucleic acids in which the sugar backbone has been replaced with morpholine rings [13, 14]. Very stable, sequence specific, and typically eliciting minimal off-target effects in the chick, MOs can be designed to block pre-mRNA splicing or translation initiation [15, 16]. However, MOs are diluted by cell division and so are ineffective over long-term culture (i.e., greater than 24 h). MOs are produced exclusively by Gene-Tools LLC and are expensive, and multiple MOs sometimes must be tried to identify one that knocks down

protein expression efficiently. Electroporated cells are traced by including a modification on the 3′ end of the MO. This modification also provides a charge (for example, fluorescein is negative, lissamine is positive) on the uncharged MO, allowing it to be electroporated (*see* **Note 1**). While the immediate effect of a splice-blocking MO can be monitored by RT-PCR, an antibody against the target protein is the best way to document knockdown, which also depends upon protein turnover. There are comprehensive guidelines available for performing controlled morpholino experiments [14, 15].

1.4 Gain of Function

Chick biologists increase endogenous gene expression levels, or express genes at ectopic times or locations, by introducing promoter-driven DNA constructs into the embryo. A variety of vectors have been created for this purpose, such as pCIG [17], pCAβ [18], and pMES [19], all of which contain the chick beta actin promoter, an internal ribosome entry site (IRES), and a bicistronic GFP reporter. Any vector used for overexpression should include a strong promoter to drive expression (enhancers can be added to make expression tissue specific, as in [20]), a fluorescent reporter such as GFP to lineage trace cells that take up the plasmid, and an IRES or viral 2A peptide to yield separate proteins [21].

1.5 Introducing Exogenous Nucleic Acid by Electroporation

At laying, a chicken embryo has thousands of cells that are too small to be microinjected directly [22]. As such, gain- and loss-of-function reagents must be introduced into chick embryos by other means, such as retroviral transfection [23] or electroporation [16, 24]. To electroporate, a bolus of nucleic acid solution is introduced adjacent to the target structure, or inside a lumen (such as the neural tube), and electrodes are positioned on either side to create an electric field that drives the nucleic acid into the target cells. This can be done directly in ovo. However, to target very early stages when the embryo is still flat (e.g., stage 4 late gastrula), embryos must be removed from the yolk in order to create an electric field perpendicular to the blastoderm and achieve electroporation. This approach is useful when working with MOs, because perdurance of stable proteins translated prior to electroporation can mask or delay the consequences of inhibiting de novo protein synthesis with the MO. As a result, to see a phenotype it is often necessary to electroporate 10–12 h before the stage to be assayed, to allow for protein turnover and/or introduce the MO before significant protein has accumulated. For example, in order to analyze gene function at stage 10, we electroporate the cells fated to become neural crest cells at stage 4 [25].

1.6 Post-incubation

After a period of incubation to the desired stage or stage equivalent, embryos and explants can be processed in order to reveal the results

of the experimental manipulation. Embryos cultured in vivo must be removed from the yolk, and all tissues must be fixed. Then, fluorescence (MOs or GFP) can be imaged, mRNA expression visualized by in situ hybridization, or protein localization determined by immunofluorescence.

2 Materials

2.1 Culturing Chicken Embryos and Explants

1. Egg incubator (humidified) set to 100 °F/38 °C (such as G.Q.F. Manufacturing model 1202).
2. Digital outlet controller (Fisher or VWR).
3. Air incubator or tissue culture incubator set to 100 °F/38 °C.
4. Fertile chicken eggs obtained from a nearby farm, or SPAFAS-specific pathogen-free eggs from Charles River (*see* **Note 2**).
5. Ringer's saline: 123.3 mM NaCl, 1.53 mM $CaCl_2$, 4.96 mM KCl, 0.809 mM Na_2HPO_4, 0.147 mM KH_2PO_4, pH 7.4 (*see* **Note 3**).
6. 70% Ethanol prepared with water.

2.1.1 In Ovo Embryo Culture

1. 3 and 1 mL syringes.
2. 25 gauge, 5/8 in, hypodermic needles.
3. 18 gauge, 1.5 in., hypodermic needles.
4. Type A India Ink (Pelikan; note that other brands can be deleterious to embryos).
5. ¾″ wide Scotch magic tape (3 M).
6. Fine, pointed scissors with a curved blade.
7. Gauze squares.
8. Beveled-edge watch glass.
9. Parafilm squares.
10. Clear packing tape (3 M; hand-tearable or 3710 packing tape; note that some tapes are deleterious to embryos).

2.1.2 Ex Ovo Embryo Culture

1. Blunt point, straight tip, standard-pattern forceps.
2. Whatman 3 mm filter paper, cut into rectangles approximately ½″ × 5/8″ with two overlapping holes punched in the center using a standard hole punch.
3. Albumen-Agar Plates: Prepare 0.51% agar in sterile 0.12 M NaCl. Heat to boiling and equilibrate to 55 °C in a water bath. Collect an equal volume of thin albumen from unincubated chicken eggs by breaking a 1 in. slit 1/3 of the way from the blunt end of the egg (use blunt-point forceps) and tipping albumen into a 50 mL conical tube. Add 10 units/mL of

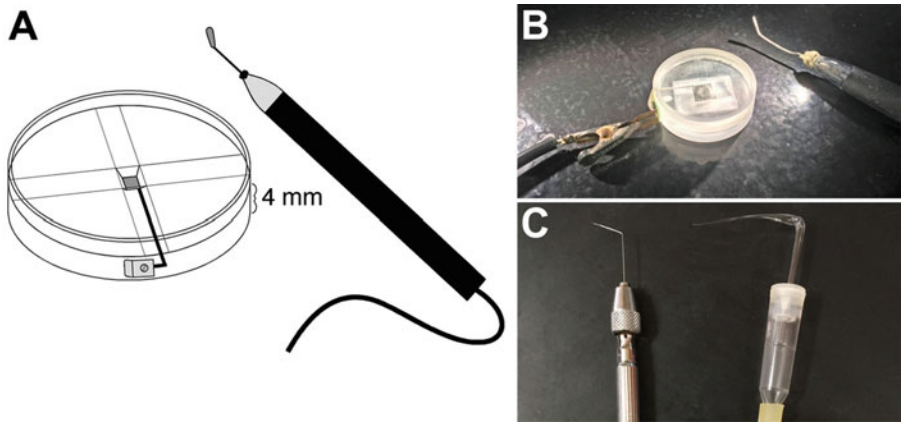

Fig. 2 Chick embryology equipment and tools. Early embryo electroporation apparatus diagram (**a**) and photograph (**b**). A platinum plate is embedded at the bottom of a 4 mm deep divet in a plastic base. Lines are etched onto the base to mark the position of the electrode plate. A wire connects the plate through the base to a flap on the side, onto which a microalligator clip attaches the bottom electrode wire. A plastic ring is attached to the top of the base, creating a dish to hold Ringer's saline. The cuvette is fixed in place under a dissecting scope using a loop of tape (visible under the dish in **b**). The top electrode is fashioned from an empty ballpoint pen casing by stringing a second wire through the casing and soldering to a 1 mm platinum wire with an end that is bent and flattened into a paddle. The wire is fixed in place with epoxy at the pen tip. Note in (**a**) that the bottom and top electrodes are not to scale. See Ref. [16] for additional details. (**c**) Useful tools. A tungsten wire sharpened to a point [26], bent at a 120° angle, and mounted in a pin holder (left) provides an efficient means to create long, straight cuts in chick embryonic tissue. Cut by pressing down through the tissue onto the bottom of the dish and sliding back and forth. A "mouth pipette" (right) is created by flame heating a glass capillary or Pasteur pipette end, which is then pulled at a 90°angle. This right-angle needle is mounted onto an aspirator assembly. The pulled needle bore can be broken off at the desired width and small pieces of tissue easily aspirated and moved without transferring much liquid

penicillin-streptomycin solution to the albumen and warm to 55 °C in a water bath. Combine the albumen and agar and swirl to mix. Pour 2 mL per 35 mm tissue culture plate. Let plates cool for 40–60 min, and then store in a sealed plastic tub at 4 °C for up to 1 week.

4. 245 mm Square bioassay dish (such as Corning 431111).

2.1.3 Explant Culture

1. Watchmaker's forceps #5 (Fine Science Tools).
2. Watchmaker's forceps #2 (World Precision Instruments).
3. Square glass baking dish.
4. Pin holder, to hold tungsten needles (Fine Science Tools; Fig. 2c, left).
5. Pin vise, to hold glass needles (Fine Science Tools).
6. Minutien insect pins (Fine Science Tools).
7. Sharpened tungsten wire: 1 in. lengths of 0.01″ tungsten wire sharpened to a point by dipping the wire tip into 1.0 N NaOH in an alkaline electrolysis bath [26] (Fig. 2c, left).

8. Glass capillary tubes (we prefer 0.8–1.1 mm diameter, 100 mm long), pulled into needles.
9. Needle puller (e.g., Sutter, Narashige, or Stoelting).
10. Mouth aspirator assembly (Sigma).
11. 35 mm Tissue culture plates.
12. Petri dishes.
13. Sylgard (Dow Corning) to coat 35 mm petri dishes (mix and polymerize Sylgard according to the manufacturer's instructions).
14. DMEM.
15. DMEM-F12.
16. N2 supplement (Thermo Fisher).
17. Fetal bovine serum.
18. Dispase: Prepare 0.15% Dispase II (Roche) in DMEM supplemented with 5 mM Hepes, pH 7.5. Freeze in 3 mL aliquots (*see* **Note 4**).
19. Ca^{2+}, Mg^{2+}-free Tyrode's saline (10×): 1.37 M NaCl, 0.27 M KCl, 4.2 mM NaH_2PO_4, with 1% glucose (*see* **Note 5**). Dilute 1× fresh before use and add 0.05% trypsin powder (Sigma).
20. Collagen: Mix 9 volumes rat tail collagen (BD Biosciences) with 1 volume 10× DMEM, add an aqueous solution of $NaHCO_3$ to a final concentration of 0.323%, and vortex well (*see* **Note 6**).

2.2 Manipulating Cell-Cell Signaling

1. 10 cm Tissue culture plate.
2. Cell line stably expressing a factor of interest or transfected with an expression construct (*see* **Note 7**).

2.2.1 Implanting Cell Pellets

3. PBS: 137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4 (*see* **Note 8**).
4. Trypsin-EDTA.
5. CellTracker CM-DiI (Thermo Fisher): Immediately before use, resuspend one 50 μg aliquot in 25 μL 100% ethanol, and then dilute in 500 μL of fresh, sterile 10% sucrose. Spin at full speed for 10 min in a microcentrifuge and transfer to a fresh microcentrifuge tube. Add 1 mL of Ringer's saline and mix. Make fresh before each use.
6. Bovine serum albumin, fraction V (Sigma).

2.2.2 Implanting Beads

1. Heparin acrylic beads (Sigma).
2. Affigel blue beads (Bio-Rad 153–7301 or 7302).

2.3 Knocking Down Gene Function

1. Morpholino (Gene Tools) resuspended at 1.0 mM in high-quality, nuclease-free water and stored at room temperature in the original vial (i.e., in glass and in the dark; *see* **Note 9**).

2.4 Gain of Function

1. Gene of interest cloned into a DNA expression vector, for example a construct containing the chick beta-actin promoter driving expression of the gene of interest, and including a bicistronic GFP reporter separated by the 2A peptide [21] or an internal ribosome entry site (IRES), such as in pCIG [17], pCAβ [18], or pMES [19].

2.5 Introducing Exogenous Nucleic Acid by Electroporation

1. Glass capillary tubes (we prefer 0.8–1.1 mm diameter, 100 mm long), pulled into needles.
2. Needle puller (e.g., Sutter, Narashige, or Stoelting).
3. Microloader pipette tips (Eppendorf).
4. Forced-air injection apparatus (e.g., General Valve Corporation Picospritzer or Harvard Apparatus PLI-100).
5. Electroporation chamber, custom made by local machine shop (see Fig. 2a, b or [16]).
6. Electroporation electrodes with a 4 mm gap (see [16, 24] for fabrication instructions).
7. Square pulse electroporator (see [16, 24] for recommendations).

2.6 Post-incubation

1. 4% Paraformaldehyde in PBS (or other fixative of choice); May be stored in aliquots at −20 °C.
2. 1 Dram vials, borosilicate glass with phenolic screw cap.

3 Methods

3.1 Culturing Chicken Embryos and Explants

3.1.1 In Ovo Embryo Culture

1. Use the Hamburger and Hamilton staging guide to determine the approximate time needed to incubate fertile chicken eggs to the stage of interest [25]. Set eggs horizontally (on their long sides, 3 rows will fit on a 2.5-dozen egg crate) and place into a humidified 100 °F/38 °C incubator for the necessary number of hours (*see* **Note 10**).
2. After incubation, let the eggs cool at room temperature for about 30 min (*see* **Note 11**). Swab the incubated eggs with 70% ethanol, taking care to maintain the egg in its original horizontal orientation as the embryo gradually floats to the top of the yolk. Once dry, place one or two 3 cm long pieces of Scotch tape across the top of the egg so that the shell may be cut without shattering.

3. Select an egg and place onto a bed of gauze on a watch glass (again, keep the position of the egg exactly as it was incubated). Carefully insert an 18 gauge needle mounted on a 3 mL syringe into the top side of the blunt end of the egg (Fig. 1a). Run the needle tip down along the blunt end of the egg, taking care not to puncture the yolk. Remove 3 mL albumen from the lowest part of the egg to drop the embryo away from the shell (*see* **Notes 12** and **13**).
4. Use a curved blade scissors to cut a 1.5–2 cm diameter window into the topmost surface of the egg, being careful to keep the lower scissor blade close to the inner surface of the shell so as not to disrupt the yolk (Fig. 1b).
5. Dilute 2–3 drops of India ink into 10 mL of Ringer's saline (about 5%). Fill a 1 mL syringe with diluted ink, outfit with a 25 gauge needle, and bend the needle to a 45° angle with the bevel pointed up. Tap and flush out air bubbles (*see* **Note 14**).
6. Insert the needle just outside the edge of the blastoderm (marked with a dotted line in Fig. 1c, and consisting of the lighter yellow, extraembryonic area opaca around the central embryonic area pellucida) and bring the needle tip up under the embryo. Dispense a small amount of ink to enhance embryo visibility (Fig. 1c). Gently shake the egg to disperse the ink, if necessary (*see* **Note 15**).
7. With the egg still sitting on the gauze/watch glass bed, look at the embryo under a microscope and determine the stage [25]. Use a pencil to mark the stage on the shell of good eggs, discard eggs that have not developed properly, or that are too young or too old (*see* **Note 12**). Add a few drops of Ringer's saline to keep the embryo moist and stretch a small piece of parafilm across the window to keep the embryo from drying out until ready to proceed.
8. After completing any manipulations (e.g., microsurgery or electroporation), gently add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
9. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.1.2 Ex Ovo Embryo Culture

1. Incubate eggs to stage 4+, usually about 25–26 h [25]. The eggs may be incubated vertically (blunt side up).
2. Wipe eggs with 70% ethanol and let cool to room temperature for 30 min (*see* **Note 11**).

3. Have ready agar-albumin plates equilibrated to room temperature ([27]; *see* **Note 16**).
4. Using a pair of blunt forceps, tap/crack/cut a 1.5–2 in. slit in the side of the egg shell about half way from the rounded end. Carefully lift off the egg shell top, and pour the yolk from the bottom shell into the palm of your gloved hand.
5. With the gloved finger of your other hand or a pair of blunt forceps, wipe and pinch off the thick albumen that adheres to the yolk and rotate the yolk until the embryo is on top (*see* **Note 17**). The embryo will appear as a light yellow circle with a clear, hourglass-shaped center. Use the edge of a pair of blunt forceps to gently wipe off the embryo to ensure that there is no thick albumen remaining attached (*see* **Note 18**).
6. Place a Whatman filter square onto the yolk so that the hole reveals the embryo. The paper will adhere to the vitelline membrane that covers the embryo. Use scissor blades to press the paper onto the yolk to ensure that it adheres well.
7. Insert a scissor blade into the yolk and cut the yolk membranes all around the paper. Grasp the embryo from one side by partially closing the scissor blades or using a #2 forceps, and gently lift one side of the embryo up and away from the yolk.
8. Place the embryo paper (dorsal) side down/yolky (ventral) side up into a dish of Ringer's saline. Gently and slowly pull the embryo back and forth through the Ringer's saline to rinse (*see* **Note 19**).
9. Place the embryo paper (dorsal) side down/yolky (ventral) side up onto an agar-albumin plate and replace the cover to maintain humidity (*see* **Note 20**).
10. Collect embryos in dishes in a humidified chamber such as a bioassay tray outfitted with moistened wipes or paper towels (*see* **Note 21**).
11. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.1.3 Explant Culture

1. Incubate several dozen eggs to the stage at which the tissue of interest is present. Use the Hamburger and Hamilton staging guide to estimate the length of incubation required [25]. The eggs may be incubated vertically (blunt side up).
2. Wipe eggs with 70% ethanol and let cool to room temperature for 30 min (*see* **Note 11**).
3. Without breaking the yolks, carefully and gently crack the eggs into a square glass baking dish a dozen at a time. The embryos will float to the top.

4. Identify the embryo. In embryos stage 12 and older, the plexus of blood vessels that surrounds the embryo marks its location. In younger embryos, find a light yellow circle with a clear, hourglass center. Using a pair of scissors, cut around the embryo to free it from the yolk (*see* **Note 22**). With the scissor blades closed, scoop under the embryo and lift, so that the embryo drapes over the closed blades (*see* **Note 23**).
5. Quickly submerge the embryo/scissor blades into a petri dish full of Ringer's saline and swish back and forth to remove the embryo. Repeat until all embryos are in the dish of Ringer's (*see* **Note 24**).
6. Lift off the vitelline membrane using a pair of forceps or a tungsten needle, and rinse the embryos using a cutoff transfer pipette. Transfer the embryos to a clean dish of Ringer's saline (*see* **Note 24**).
7. Tissues may be isolated through a variety of methods. Explants may be manually dissected from superficial structures (for example a neural fold) using glass or tungsten needles, or a pair of spring scissors.
8. To facilitate dissection of adherent tissues, minutien insect pins may be used to fasten embryos to a Sylgard-coated dish in calcium and magnesium-free Tyrode's saline +0.05% trypsin. Over time, this solution causes tissues to release from one another, and allows the tissue of interest to be more easily dissected free with pulled glass capillary needles or sharpened tungsten wire.
9. Another option is to cut blocks of tissue using a tungsten needle bent at a ~120° angle (Fig. 2c, left) by pressing the bottom edge of the wire through the tissue against the bottom of the dish and sliding back and forth (*see* **Note 25**). This tissue block can be incubated in dispase to cleave extracellular matrix proteins and gently dissociate tissues from one another (*see* **Note 26**). Dispase-treated tissue can then be "triturated" (pipetted in and out) against the side of the dish with a fire-polished Pasteur pipette to separate adhering layers.
10. Trypsin or dispase-treated tissue explants should be rinsed immediately in several changes of ice-cold media (DMEM or DMEM-F12) + 10% fetal bovine serum (*see* **Note 27**).
11. Spread 6 μL of collagen onto the bottom of a tissue culture dish (*see* **Note 28**). Cover the dish and allow the collagen to set (it will become opaque), but do not let it dry out.
12. Place the tissue/cells to be cultured on the collagen bed. Transfer as little solution as possible (*see* **Note 29**). Add 4 μL more collagen over the tissue and allow to set.

13. Cover the collagen-embedded tissue with an appropriate culture medium (we use DMEM-F12 + N2 supplement for neural crest cells) and culture at 37 °C with CO_2 for the number of hours necessary to reach the desired stage equivalent (the stage the embryo from which the explant was dissected would have been after the incubation) [25].

3.2 Manipulating Cell-Cell Signaling

3.2.1 Implanting Cell Pellets

1. Prepare a confluent 10 cm plate of a stable cell line or cells transfected with an expression construct using standard tissue culture protocols. Remember to prepare control cells as well (e.g., cells transfected with a control vector).
2. Remove the media from the cells and wash the plate with 10 mL of PBS. Aspirate well. Add 1 mL of trypsin-EDTA and rock/rotate the plate to cover the entire surface. Aspirate excess trypsin. Incubate for ~3–5 min, as needed until cells break free.
3. Collect the cells by rinsing with 10 mL of media + 20% fetal bovine serum. Transfer cells to a 50 mL conical tube. Pipette in and out to achieve a single-cell suspension, and then allow cells to recover for 30 min in the tissue culture hood, swirling occasionally to keep in suspension.
4. Bring the volume to 50 mL with PBS or Ringer's saline. Gently collect cells by centrifugation at 225 rcf for 5 min.
5. Carefully aspirate the supernatant and resuspend cells in 300 μL of DiI solution. Incubate for 15 min in the hood at room temperature.
6. Bring the volume to 50 mL with PBS or Ringer's. Gently collect cells by centrifugation at 225 rcf for 5 min. Aspirate supernatant.
7. If cells will be injected, resuspend cells in 1 mL of Ringer's saline + 0.1% BSA. Transfer to a 1.7 mL microcentrifuge tube, centrifuge at 300 × *g*, and aspirate supernatant. Resuspend pellet in an equal volume of Ringer's saline + 0.1% BSA by tapping and flicking the tube. Tube can be stored on ice. To inject, back load 2 μL of thoroughly resuspended cells into a pulled glass capillary needle using a microloader pipette tip. Air pressure inject groups of cells into a region of interest (e.g., the lumen of the neural tube, the head mesenchyme; *see* **Notes 30** and **31**).
8. If cells will be surgically grafted, resuspend cells from **step 6** in an equal volume of complete culture medium by tapping and flicking. Pipette 20–50 μL drops of cell suspension onto the underside of a tissue culture plate lid. Fill the dish itself with media or PBS (for humidity), invert the lid to cover the dish, and incubate the "hanging drop" at 37 °C in a CO_2 incubator for 24 h. The cells will form spheroids that can be grafted into

host embryos using a pair of forceps to insert them through incisions cut with sharpened tungsten wire.

9. After either **steps 7** or **8**, add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
10. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.2.2 Implanting Beads

1. Obtain Heparin Acrylic or Affigel Blue beads according to the application (*see* **Note 32**).
2. Wash the beads three times by rolling/moving them through a series of PBS drops on the lid of a tissue culture plate.
3. Place a drop of protein diluted in PBS + 0.1% BSA at the center of a 35 mm tissue culture dish (*see* **Note 33**). Place additional drops of PBS around the bottom of the dish for humidity.
4. Select a bead and place it on a dry area of the plate. Move the bead back and forth to draw out excess PBS. This step is important so that you do not transfer PBS along with the bead and dilute the protein.
5. Place the dried bead into the protein drop and incubate for 2 h at room temperature to 4 °C overnight.
6. Wash the bead by rolling/moving it through two or three drops of PBS.
7. Implant the bead into a region of interest in the embryo. Typically a slit must be prepared with a sharpened tungsten needle, and the bead pushed into the slit with forceps (*see* **Note 34**).
8. Add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
9. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.3 Knocking Down Gene Function

3.3.1 Morpholino Oligonucleotides (MOs)

1. Find your target gene of interest in the latest chick genome assembly using www.ensembl.org.
2. Decide on the type of MO to use. A translation blocking MO anneals over the translation start site to sterically block the translation initiation complex. A splice blocking MO anneals over a splice acceptor or splice donor site in a pre-mRNA to disrupt splicing and induce exon skipping, causing a frameshift

Table 1
Issues to consider when selecting the type of morpholino to use in your experiments

Translation blocking MO	Splice blocking MO
Sterically blocks the translation initiation complex to prevent translation	*Blocks a splice donor or acceptor site in the pre-mRNA to cause exon skipping, a frameshift, and premature stop*
A good choice when: – Sequence annotation includes a high-confidence translation start site without alternate/cryptic translation start sites – A specific start site/isoform is to be targeted	*Advantages:* – The efficacy of the MO can be assayed by PCR amplification of the targeted exon and the splicing outcome determined by sequencing the product – When MOs are designed against a splice junction acceptor and donor site, a "dose-synergy" phenotype generated by sub-phenotypic doses of each MO is evidence of specificity [14]
A challenging approach when: – There is no antibody available for the target protein in order to assay knockdown efficiency	*Not an option when:* – The gene contains no introns – The first coding exon is very long – Length of initial exons is divisible by three so splicing is in frame even when an exon is skipped

and a premature stop. A variety of factors will determine which MO type is best suited to an application (Table 1; see also guidelines in [14]).

3. Decide on the type of 3′ end modification to add. Unmodified morpholinos have no charge and thus will not be transfected into cells by electroporation. Addition of a 3′ modification provides a charge to allow for electroporation as well as a means to visualize targeted cells directly (fluorescein, lissamine) or indirectly (biotin). Different modifications carry different charges (fluorescein and biotin have a net negative charge, while lissamine has a net positive charge; *see* **Note 1**), which dictates the polarity of the electric field necessary for transfection.
4. www.gene-tools.com provides design recommendations and offers a free design service.

3.4 Gain of Function

1. Use standard molecular biology methods to subclone your gene of interest into an expression vector [28]. If the gene is not already cloned, we design primers based upon the genome sequence and use a high-fidelity DNA polymerase to PCR amplify the gene from chick embryo cDNA, following standard molecular biology methods [28]. It is important to limit the amount of untranslated sequence that is included with the gene of interest, as untranslated sequence can cause genes to be expressed less efficiently.

2. Prepare plasmid DNA that is endonuclease free and concentrated enough to yield 1 μg/μL to 4 μg/μL dilutions for electroporation (1 μg/μL is usually a good starting point; *see* **Note 35**).

3.5 Introducing Exogenous Nucleic Acid by Electroporation

3.5.1 In Ovo Electroporation

1. Prepare windowed eggs as in Subheading 3.1.1.
2. Use a microloader tip to back-fill a glass needle with 2–5 μL of plasmid or MO. Place the needle in the needle holder of a forced-air injection apparatus (*see* **Note 30**). Break off the very tip of the needle and expel any air remaining in the tip. Test and adjust the injection time until only a small drop is produced.
3. Introduce a bolus of plasmid or MO adjacent to the tissue to be electroporated (*see* **Notes 31**, **36**, and **37**). Best results will be obtained by injecting into a lumen (e.g., the interior of the neural tube) that will act as a reservoir to contain the injected material and limit diffusion. Keep in mind that negatively charged DNA and the negative charge of a fluorescein modification on the MO (MOs themselves are uncharged) will enter cells in the direction of the positive electrode. Positively charged lissamine-modified MO will enter cells in the direction of the negative electrode. *See* **Notes 1** and **38** and [16, 24] for additional pointers.
4. Moisten the electrodes with Ringer's saline and place them 4 mm apart on either side of the embryo, with the tissue to be electroporated located between the injected material and the relevant electrode (positive for DNA and fluorescein-conjugated MOs, negative for lissamine-conjugated MOs). The degree to which the electrode contacts the embryo surface and the angle between the electrodes will determine the extent and direction of electroporation (*see* **Note 39**).
5. For ~10-somite embryos, apply five, square-wave 20 volt, 50-ms pulses with 100-ms intervals between pulses (*see* **Note 40**).
6. Let the embryo recover for 1–2 min, then gently place a few drops of Ringer's saline onto the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (Fig. 1d). Make sure that the egg is well sealed or the embryo will dry out.
7. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.5.2 Ex Ovo Electroporation

1. Prepare embryos on agar-albumin plates as in Subheading 3.1.2.

2. When ready to electroporate, excess yolk that is adhering to the embryo must be removed as it will impede visualization as well as electroporation. Tilt the agar-albumin plate toward you at a 45° angle and use a transfer pipette to drip Ringer's saline onto the elevated side of the plate (avoiding dripping directly onto the embryo) so that Ringer's saline washes over the embryo to moisten it. Then, either tip the dish back and forth to further clean yolk from the embryo and use the transfer pipette to aspirate the Ringer's wash from the dish or use a pair of forceps to lift the embryo from the plate and drag it gently back and forth through a dish of clean Ringer's saline.
3. Place the embryo paper (dorsal) side down/yolky (ventral) side up in an electroporation cuvette (Fig. 2a, b) containing clean Ringer's saline.
4. Using published fate maps, determine the location of the precursors of the cell type to be targeted in a stage 4+ embryo [29–32]. Use the cuvette alignment lines to orient the embryo so that this target region lies over the center of the bottom electrode (*see* **Note 41**).
5. Use a microloader tip to back-fill a glass needle with 2 μL of diluted plasmid or MO. Place the needle in the needle holder of a forced-air injection apparatus (*see* **Note 30**). Break off the very tip of the needle and expel any air remaining in the tip. Test and adjust the injection time until only a small drop about 1×–1.5× the width of the primitive streak is produced (*see* **Note 42**).
6. Gently insert the needle through the embryo from the top-facing ventral side into the subvitelline space and expel a puddle of plasmid or MO adjacent to the cells of the blastoderm to be electroporated (*see* **Note 31**). Embryos may be targeted unilaterally (useful when characterizing a knockdown phenotype, as the untargeted internal control establishes the baseline for that embryo), or on both sides (useful when collecting tissue for other manipulations) by varying the size and location of the puddle.
7. Before the injected solution has a chance to disperse, place the upper electrode into the Ringer's saline directly over (but not touching) the region of the embryo containing the injected solution (top and bottom electrodes should be ~4 mm apart). If the paddle of the top electrode is not covered with Ringer's saline, add more. Ideally, the upper electrode should be placed symmetrically so that both the targeted (injected) and untargeted control sides are exposed (*see* **Note 43**).
8. Apply five, square-wave 7 volt 50-ms pulses with 100-ms gaps between pulses (*see* **Note 40**).

9. Transfer the embryo from the electroporation cuvette back to the agar-albumin plate, paper/embryo dorsal side down.
10. Incubate to the desired stage in a humidified 100 °F/38 °C incubator [25].

3.6 Post-incubation

3.6.1 In Ovo Embryo Processing

1. When the embryo has reached the desired stage [25], cut the tape sealing the egg window and add a few drops of Ringer's saline to the embryo to moisten the surface (*see* **Note 44**).
2. Use a pair of scissors to cut around the embryo. Lift the embryo from the yolk by grasping a cut edge with a pair of forceps, or by scooping and lifting under the embryo with a pair of closed scissor blades (*see* **Note 45**).
3. Place the embryo into a petri dish with Ringer's saline. Rinse yolk from the embryo, lift off and discard the vitelline membrane, and trim extraembryonic tissues (*see* **Note 25**).
4. Fix embryos with 4% paraformaldehyde and store in 1 dram vials according to standard protocols for immunocytochemistry or in situ hybridization [33] (*see* **Notes 46** and **47**).

3.6.2 Ex Ovo Embryo Processing

1. Embryos may be fixed while still attached to the paper in order to maintain a flat conformation. To do this, remove embryos from agar-albumin plates and place them embryo side up in a dish of Ringer's (keep them sorted in separate dishes according to the construct that was electroporated). Spread them out around the bottom of the dish.
2. Alternatively, the embryo may be lifted off of the filter paper for more compact storage (e.g., in 1 dram vials). Place the embryo into a petri dish of Ringer's saline with the paper side down. Find a place where the membranes are rolling up or detaching. With a pair of forceps, gently peel the embryo away (the vitelline membrane will remain attached to the paper) and trim extraembryonic tissues (*see* **Notes 25** and **48**).
3. Fix embryos with 4% paraformaldehyde and store in 1 dram vials according to standard protocols for immunocytochemistry or in situ hybridization [33] (*see* **Notes 46**, **47**, and **49**).

3.6.3 Explant Processing

1. After incubation, tissue embedded in collagen can be fixed and processed for immunocytochemistry or in situ hybridization using standard protocols [33]. The collagen may be left attached to the culture dish for staining, or may be gently lifted off with a pair of forceps and processed as a single mass (*see* **Note 50**).

4 Notes

1. Although lissamine effectively traces the MO and allows for electroporation, because it is positively charged, it cannot be co-electroporated with negatively charged DNA in order to assay rescue of a knockdown phenotype (as a specificity control). Thus, lissamine modification of MOs is not recommended.
2. Eggs can be stored at 13–20 °C for up to 1 week before use, although fertility (i.e., number of viable embryos) will be highest when eggs are fresh and will decline over time. An old refrigerator outfitted with a WINE-STAT (Walnut Creek, CA) makes an inexpensive egg-storage cooler. Eggs may also be placed in a cool corner of the lab.
3. 1 L of Ringer's saline is prepared by dissolving 7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl, 0.115 g Na_2HPO_4, and 0.02 g KH_2PO_4 in ~900 mL of water, adjusting pH to 7.4, and adding H_2O up to 1 L. Filter sterilize; do not autoclave.
4. Excess DMEM plus HEPES may be stored in 50 mL aliquots at −20 °C for later use.
5. 1 L of 10× Ca^{2+}, Mg^{2+}-free Tyrode's saline is prepared by dissolving 80 g NaCl, 2 g KCl, 0.5 g NaH_2PO_4, and 10 g glucose in H_2O up to 1 L. Filter sterilize; do not autoclave.
6. 100 µL of collagen is prepared using 90 µL of rat-tail collagen, 10 µL of 10× DMEM, and 4.5 µL of 7.5% $NaHCO_3$. The collagen mix can be stored for short periods on ice before use. The solution should be light pink and set in 15–20 min at room temperature. More (or less) $NaHCO_3$ can be added if this is not the case.
7. We have successfully implanted cell pellets of many different cell lines.
8. 1 L of PBS is prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in ~900 mL of water, adjusting pH to 7.4, and adding H_2O up to 1 L. Autoclave. PBS can also be prepared as a 10× stock and diluted to 1× with sterile water before use.
9. Storing resuspended morpholino in plastic and at cold temperatures may result in precipitation and morpholino loss. If aliquots need to be made, they should be freeze-dried according to the protocol on the Gene Tools website (www.gene-tools.com), and individually resuspended before each use.
10. Incubators may be placed on digital outlet controller timers in order to start eggs at inconvenient times (e.g., the middle of the night).

11. Yolks are more fragile and break easily when hot.
12. Before starting, have ready a bag to discard damaged or unneeded eggs and a beaker to contain removed albumen. Egg waste should be disposed as required by your institution.
13. It is typically not necessary to apply scotch tape over the syringe needle hole as the egg usually seals itself; however, tape should be applied if the hole is extensive. Alternatively, if the syringe needle hole is far enough toward the top of the egg, it may be used as a starting point to cut the access hole in the next step (Subheading 3.1.1, **step 4**).
14. If bubbles are injected into the yolk along with diluted ink, they will eventually be resorbed and will not alter development; however, bubbles make it more difficult to visualize the embryo.
15. Over time the ink may disperse within the yolk, and the embryo may be re-inked. When possible, reinsert the needle through the same hole next to the blastoderm.
16. While collecting and working with embryos, agar-albumin plates can be stored in a bioassay tray to keep them protected and organized.
17. Make sure that the gloved finger you use to wipe the yolk is wet with albumen and not dry, or you will break the yolk.
18. If albumen remains on the yolk, the paper will not stick well, the embryo will detach, and development will be abnormal or arrested.
19. Be gentle and patient. Do not wash the embryo vigorously, and be sure that the embryo does not detach from the paper. Both over- and underwashing lead to problems with viability.
20. Embryos are stable on agar-albumin plates at room temperature for about 2–4 h. Depending on the size of your embryos/filter papers, two to three embryos may be cultured on a single agar-albumin plate.
21. After culture, bioassay dishes can be washed and reused multiple times.
22. When cutting the yolk membranes, make your first cuts along the side of the embryo that is lowest on the yolk. The contents of the yolk will spill out once it is broken; thus if you cut the highest point first, the embryo will be pulled under the yolk.
23. Embryos stage 10 and younger are small and very fragile. Paper disks (as described in Subheading 3.1.2) may be used to support young embryos during isolation from the yolk.
24. Chick embryos will stick to untreated plastic dishes and transfer pipettes. To avoid sticking, rinse unused plastic with Ringer's saline that is milky with egg yolk debris (for example, Ringer's

saline in which embryos have been rinsed, or into which a small amount of egg yolk has been added). After coating the plastic with yolky Ringer's, discard and rinse with fresh Ringer's saline, and then proceed.

25. Bent tungsten needles (Fig. 1c) are also convenient for trimming excess extraembryonic membrane after embryo fixation.
26. To isolate trunk neural tubes, we incubate stage 14–15 caudal trunk segments in dispase for 15 min on ice, and then 10 min at 37 °C, until tissues begin to fall apart. Dispase incubation conditions will need to be optimized for each tissue type.
27. Neural crest cell cultures grow best in DMEM-F12 media. However, to rinse away enzyme, either DMEM or DMEM-F12 is acceptable.
28. The collagen should be spread only a little. It should not be too thin, nor should it be significantly domed. More collagen can be used if larger explants are cultured.
29. With practice, a glass needle bent in a ~90° angle (created by holding a capillary tube or Pasteur pipette tip in a flame and pulling in a perpendicular direction) fitted onto an aspirator assembly (shown in Fig. 1c) provides a controlled means to move small tissue explants from one dish/location to another while transferring very little liquid.
30. Injections can be performed using a commercial forced-air microinjection instrument, or a homemade apparatus with a capillary microinjection holder (such as Warner MP-S10 T or similar) outfitted onto tubing attached to compressed air with tubing incorporating a "Y" connector (covering the open branch of the "Y" will force air out of the capillary tip). If the material being injected is not too dense/viscous a mouth aspirator assembly can also be used.
31. We generally hold the needle assembly in our hand to inject, but a micromanipulator can also be used to hold the needle steady.
32. Affigel blue beads work for a variety of proteins, while heparin acrylic beads work well for growth factors that bind heparin. Affigel blue beads are "stickier," while heparin acrylic beads can be easier to manipulate. Deciding which bead to use is a combination of the factor being released, preference, and empirical evidence/experience.
33. The protein concentration used depends upon the factor of interest and must be determined empirically. Generally, FGFs, Shh, and Noggin are typically used at 1 mg/mL, while BMPs can be used at lower concentrations, for example 0.1 mg/mL.

34. If your bead will not stay in place, try using a smaller bead. Heparin acrylic beads can also be chipped and a smaller fragment used instead.
35. DNA can be ethanol precipitated prior to electroporation if more concentrated samples are needed; however, be sure to include a 70% ethanol wash to remove salt as it can interfere with electroporation.
36. If the injected solution dissipates rapidly, sterile 10% sucrose can be added at 1:4 or 1:1 to make the solution more viscous and less easily displaced. Be sure to account for the extra volume when preparing the DNA or MO dilution.
37. Although the fluorescein or lissamine modifications give the MO solution color, 2% vegetable dye (FD&C Blue, Spectra Colors Corporation) can be added to the MO at 1:10–1:20 for improved visibility of the injected solution. Fast Green is not recommended because it can inhibit the uptake of the MO [34].
38. When mixed, MO and DNA each facilitates the electroporation of the other. This must be kept in mind when attempting to rescue a MO knockdown phenotype by co-electroporating a DNA expression construct, but can also be used to increase electroporation efficiency. For example, adding 0.3 μg/μL DNA (any non-biologically active DNA will do) to the MO can vastly improve its electroporation efficiency. Likewise, including low concentrations of a non-targeting MO can increase the efficiency of DNA electroporation as well.
39. The tips, bends, or arms of the electrodes may be placed on the embryo for electroporation. The angle of electroporation is changed by placing more pressure on one electrode than the other.
40. The number of pulses and voltage can be adjusted to optimize survival and targeting efficiency for your tissue and age of embryo. Generally speaking, younger embryos must be electroporated with lower voltages.
41. Ensure that the embryo is clean so that large amounts of yolk are not blocking the lower electrode or covering the embryo.
42. The drop size produced will determine how localized versus dispersed the solution that is introduced becomes, and can be varied according to preference and the requirements of the experiment. Multiple injections can also be performed to increase the size of the puddle.
43. To ensure that both sides of the embryo are treated equally, we place the upper electroporation paddle perpendicular to the primitive streak and over both sides of the embryo, even if only one side is being transfected. Thus, both sides are

electroporated, but there is a targeted and an untargeted side for comparison to control for electroporation.

44. The surface of the embryo will be very sticky without being moistened first.
45. It may be necessary to grasp one edge of the embryo with a pair of forceps while you cut so that the embryo isn't pulled under by spilling yolk.
46. Fixation can attenuate GFP fluorescence; thus embryos electroporated with GFP-expressing constructs may need to be imaged prior to fixation or after a brief initial fixation.
47. It is a good idea to image fluorescein-modified MO-electroporated embryos before subsequent staining protocols as the fluorescence quickly fades. Keep the embryos in the dark at all times to minimize this.
48. Embryos may also be removed from the filter papers in this manner after fixation and before storing in a 1 dram vial.
49. Ex ovo culture can result in nonspecific midbrain neural tube closure defects. By electroporating only one side of the embryo, phenotypes can be interpreted (by comparing to the untargeted side) as long as appropriate controls are included.
50. Occasionally the collagen gives high background staining, in which case it is necessary to dissect the pieces of tissue from the collagen and stain them individually (this should be done right before staining as small explants can be very difficult to retain and track).

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Chapter 7

Identifying Protein-DNA and Protein-Protein Interactions in Avian Embryos

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Abstract

The chick embryo is a powerful model for experimental embryology due to its accessibility, sturdiness, and ease of manipulation. Here we describe protocols for analysis of protein-DNA and protein-protein interactions in tissues and cells isolated from the developing chick. These assays are aimed at the identification of interactions between transcription factors and regulatory elements in the genome, and, in combination with functional assays, can be used for the delineation of gene regulatory circuits.

Key words Chick embryo, Protein-DNA interactions, Protein-protein interactions, Chromatin precipitation (ChIP), Co-immunoprecipitation (Co-IP)

1 Introduction

Characterizing molecular interactions is an important step in the study of the regulatory programs that orchestrate embryonic development. In particular, the identification of protein-DNA and protein-protein complexes that are present in specific spatial domains of the embryo can inform upon the mechanisms controlling patterning and morphogenesis [1]. There are, however, considerable challenges in performing such assays in embryonic tissue. Most of the established protocols aimed at testing protein-DNA and protein-protein interactions require large amounts of starting material. Here we present protocols for two gold standard biochemical assays in the chick embryo: *chromatin immunoprecipitation* (ChIP) [2, 3] for identification of binding of transcription factors to DNA targets, and *co-immunoprecipitation* (Co-IP) [4] for characterization of protein complexes. These protocols have been optimized for a relatively low input, requiring a small number of embryos (or small amounts of embryonic tissue) as starting material.

Both methods are based on the immunoprecipitation of a target protein via specific antibodies that have been complexed to

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_7,

magnetic beads. In the ChIP assay, cross-linked fragments of DNA-protein complexes are immunoprecipitated from the cell lysates using a protein-specific antibody. Associated DNA fragments can subsequently be amplified through qPCR analysis using primers for the sequence of interest. In our Co-IP protocol, we utilize ex ovo electroporation [5] to drive the expression of two proteins of interest. The bait protein is fused to an Avi tag, which can be isolated using streptavidin-coated beads upon in vivo biotinylation mediated by the BirA enzyme. Cofactors bound to the bait protein are subsequently identified by western blotting. We routinely perform these experiments with tissue explants obtained through microdissection, although cells obtained through FACS can also be used. We find that the chick embryo, which is easy to obtain and dissect, is a convenient vertebrate model organism for biochemical studies.

2 Materials

2.1 Chromatin Immunoprecipitation: ChIP-qPCR

1. Formaldehyde stock solution: 37% Formaldehyde.
2. Glycine stock solution: 1 M Glycine.
3. DTT stock solution: 1 M DTT.
4. Sucrose stock solution: 1.5 M Sucrose.
5. PMSF stock solution: 0.2 M PMSF, in EtOH (*see* **Note 1**).
6. Protease inhibitor tablet (Complete, Mini EDTA-free)—Roche.
7. Protease inhibitor stock solution: Prepare 7× stock solution by dissolving 1 tablet in 1.5 ml of ddH_2O (*see* **Note 2**).
8. Tris–HCl, pH 7.5 stock solution: 1 M Tris–HCl, pH 7.5.
9. Tris–HCl, pH 8.0 stock solution: 1 M Tris–HCl, pH 8.0.
10. Hepes-KOH stock solution: 1 M Hepes–KOH, pH 8.
11. NaCl stock solution: 5 M NaCl.
12. LiCl stock solution: 5 M LiCl.
13. $CaCl_2$ stock solution: 0.1 M $CaCl_2$.
14. 10× TE: 100 mM Tris–HCl pH 8, 10 mM EDTA.
15. SDS stock solution: 20% SDS.
16. EDTA stock solution: 0.5 M EDTA pH 7.5.
17. Triton X-100 stock solution: 10% Triton X-100.
18. NP-40 stock solution: 10% NP-40.
19. Accumax—Innovative Cell Technologies.
20. Dynabeads™ Protein G beads—Invitrogen.
21. DynaMAg™-2 magnet—Invitrogen.

22. RNase A.
23. Proteinase K.
24. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
25. 2 mL Small glass Dounce homogenizer set (pestle B).
26. Ringer's solution (0.125 M sodium chloride, 1.5 mM calcium chloride, dehydrate, 5 mM potassium chloride, 0.8 mM sodium phosphate, dibasic).
27. PBS (137 mM NaCL, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).
28. Blocking solution: 0.5% BSA in PBS, filtered.
29. Nuclei extraction buffer (prepared fresh): 0.5% NP-40, 0.25% Triton X-100, 10 mM Tris–HCl, pH 7.5, 3 mM CaCl2, 0.25 M sucrose, protease inhibitor tablet (1 mini tablet/10 mL of buffer), 1 mM DTT, and 0.2 mM PMSF.
30. Lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 7.5, 1× Protease Inhibitor.
31. Dilution buffer: 0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
32. Triton dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl. 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
33. RIPA (washing buffer): 50 mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-deoxycholate.
34. TE/NaCl: 10 mM Tris–HCl, 1 mM EDTA pH 8.0, 50 mM NaCl.
35. Elution buffer: 1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0.

2.2 Co-immunoprecipitation: Co-IP

1. DTT stock solution: 1 M DTT.
2. PMSF stock solution: 0.2 M PMSF, in EtOH (*see* **Note 1**).
3. Protease inhibitor tablet (Complete, Mini EDTA-free)—Roche.
4. Protease inhibitor stock solution: Prepare 7× stock solution by dissolving 1 tablet in 1.5 ml of ddH_2O (*see* **Note 2**).
5. Ringer's solution (0.125 M sodium chloride, 1.5 mM calcium chloride, dehydrate, 5 mM potassium chloride, 0.8 mM sodium phosphate, dibasic).
6. PBS (137 mM NaCL, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

7. 1 mL Syringe and 27.5 gauge needles.
8. Hepes-KOH stock solution: 1 M Hepes-KOH pH 8.
9. NaCl stock solution: 5 M NaCl.
10. 1% BSA in PBS, filtered.
11. Dynabeads™ MyOne™ Streptavidin T1—Invitrogen.
12. DynaMAgTM-2 magnet—Invitrogen.
13. LDS Sample Buffer (4×)—Life Technologies.
14. Sample Reducing Agent (10×)—Life Technologies.
15. Hypotonic buffer: 10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
16. IP extraction buffer: 20 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 20% glycerol, 420 mM KCL, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors 1× (added just before use).
17. Modified RIPA buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40.

3 Methods

3.1 Chromatin Immunoprecipitation: ChIP-qPCR

Here we describe our approach for performing ChIP-qPCR in tissue explants dissected from chick embryos (Fig. 1). The protocol is standardized for small amounts of tissue; we found that most abbreviated protocols result in loss of sensitivity and thus are not suitable for small amounts of embryonic cells. Wild-type embryos are collected from eggs and dissected. Embryonic explants are dissociated to a single-cell suspension with Accumax, and cross-linked with formaldehyde. We use a Diagenode Bioruptor for chromatin shearing, although Covaris can be used as well. For immunoprecipitation, we found that Protein G Dynabeads give us the best results, even for rabbit antibodies. Throughout the protocol, it is essential to employ careful pipetting during the washes to minimize loss of material.

3.1.1 Embryo Dissociation and Cross-Linking

1. Dissect embryos in Ringer's solution (*see* **Note 3**).
2. Transfer dissected tissue (~50–100,000 cells) to a microcentrifuge tube.
3. Quickly wash embryos with Ringer's to remove yolk and other debris. Spin 2′ at 200 rcf to collect the tissue at the bottom of the tube.
4. Rinse quickly with 300–500 μL Accumax to remove excess salt from Ringer's.

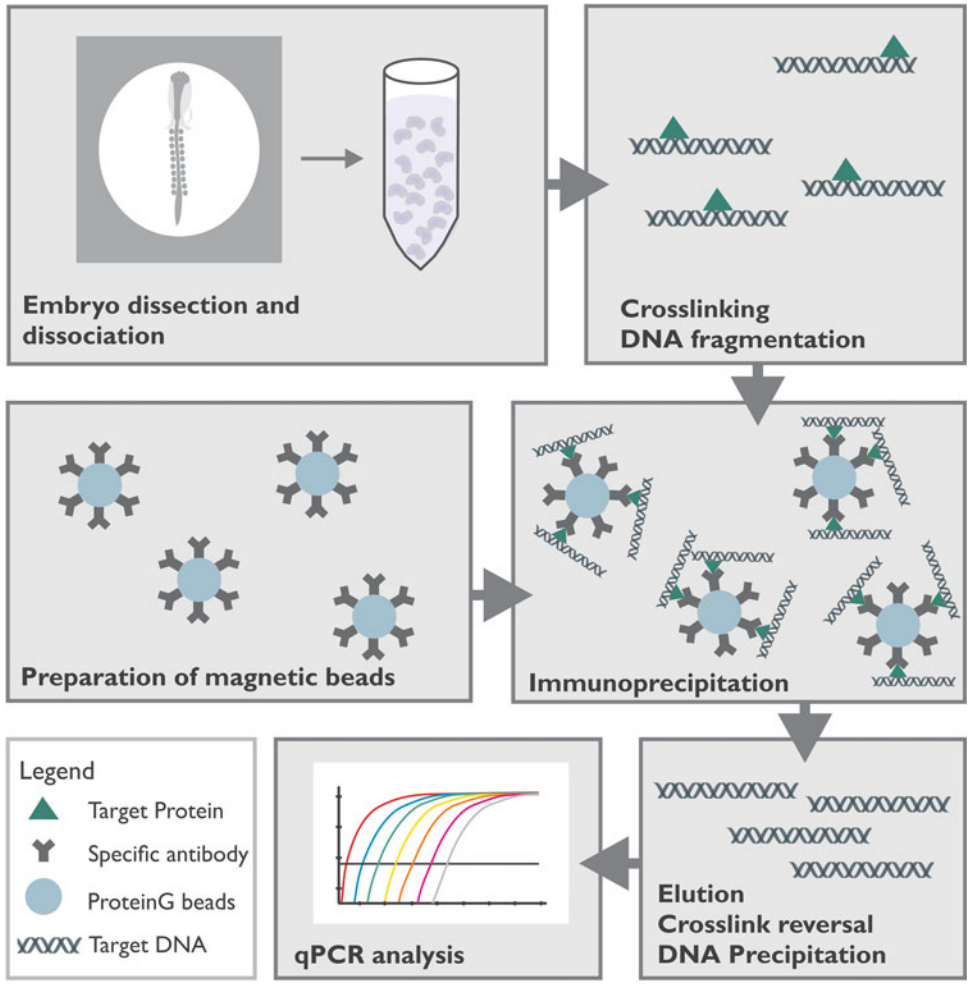

Fig. 1 Schematic overview of the ChIP-qPCR protocol

5. Add 973 μL of Accumax and incubate the tissue at RT for 20–30′, rocking at orbital shaker. During this period, gently pipette the tissue up and down to help tissue start dissociate.
6. Add 27 uL of 37% formaldehyde to each tube. Cross-link for exactly 10′ at RT, rocking.
7. Add 143 μL of cold 1 M glycine to each tube to quench the cross-linker. Make sure that the glycine is properly mixed with the cell suspension.
8. Immediately spin down the cells for 4′ at 2000 rcf, 4 °C.
9. Gently resuspend cells with ice-cold PBS supplemented with protease inhibitors (1× protease inhibitor, 10 μL 1 M DTT, and 20 μL 0.2 M PMSF). During washes, samples should be kept on ice to avoid protein degradation.
10. Spin down the cells for 4′ at 2000 rcf, 4 °C.

11. Repeat washes two more times. After the last wash, remove all of the PBS and immediately freeze the pellet with liquid nitrogen. Store cells at −80 °C.

3.1.2 Lysis and Chromatin Fragmentation

1. Resuspend pellet (fresh or frozen) in 1 mL nuclei extraction buffer and transfer the sample to a 2 ml small glass Dounce homogenizer that has been kept on ice (*see* **Note 4**).
2. Re-homogenize with 10 strokes using Pestle B. This will disrupt the cellular walls and help expulse the nuclei into the solution.
3. Transfer the sample to a 1.7 mL tube. Spin for 1′ at maximum speed at 4 °C.
4. Remove the supernatant and wash the pellet once with ice-cold PBS supplemented with protease inhibitors.
5. On ice, resuspend the pellet in 140 μL lysis buffer.
6. Pipette up and down to release the cross-linked chromatin into the solution. Allow to lay on ice for 10–20′ (this time can be extended to 1 h).
7. Add 2 volumes (280 μL) of dilution buffer.
8. Shear the chromatin to approximately 200 to 1000 bp fragments using a sonicator (for Diagenode Bioruptor, set the equipment at "High," 30" ON, 30" OFF, for 11′). The samples should be kept in ice bath throughout the process to avoid overheating and foaming.
9. Add 42 μL of 10% Triton X-100 to the sonicated chromatin. Centrifuge the sonicated solution at 20,000 rcf for 10′ at 4 °C.
10. Transfer the supernatant to a fresh tube (~400 μL) without disturbing the pellet. The supernatant contains the sheared chromatin, and the pellet can be discarded.

3.1.3 Preparation of Magnetic Beads

1. Gently homogenize the bead suspension extensively. For each IP (protein-specific antibodies and negative control), add 100 μL of magnetic beads to a microfuge tube.
2. Add 1 mL blocking solution.
3. Collect the beads using magnetic stand. Let the beads separate for ~3′ and remove supernatant.
4. Wash beads in 1 mL blocking solution two more times. Each time resuspend the beads in the blocking solution by gentle tapping on the tube and allow the beads to wash while nutating for 2–3′. When removing the liquid, leave the tubes for a minimum of 3′ on the magnetic stand, to collect all the beads.
5. Resuspend beads in 250 μL blocking solution and add 10 μL of antibody for each IP (*see* **Note 5**).
6. Incubate overnight on the rotisserie in the cold room.

7. Next day, wash beads as described above (three times in 1 mL block solution).
8. Resuspend Dynabeads in 100 μL of blocking solution.

3.1.4 Chromatin Immunoprecipitation

1. Dilute the sheared chromatin (**step 10** in Subheading 3.1.2) to 800 μL (for ChIP with two antibodies) or 1200 μL (for ChIP with three antibodies) with triton dilution buffer.
2. Save an aliquot of 20 μL of the diluted chromatin and keep at −80 °C to be used as input sample.
3. Add ~400 μL of the diluted chromatin to antibody/magnetic bead mix from **step 8** in Subheading 3.1.3.
4. Incubate O/N on rotisserie in the cold room.

3.1.5 Washing, Elution, and Cross-Link reversal

1. Prechill the magnetic stand (leave at −20 °C O/N).
2. All washing steps should be done in a 4 °C cold room. Let tubes sit for at least 3′ in magnetic stand to collect the beads. Remove supernatant.
3. Add 1 mL of ice-cold RIPA to each tube. Remove tubes from magnetic stand and tap gently to resuspend beads. Leave tubes on the nutator for 1–2′ for more efficient wash. Replace tubes in magnetic stand to collect beads. Remove and discard supernatant.
4. Repeat this wash seven more times.
5. Wash once with 1 mL of TE/NaCl.
6. To reduce the nonspecific background, after 3′ wash, change to chromatin/bead suspension to a new chilled tube. Separate the beads using the magnetic stand and remove the supernatant as previously.
7. Spin the tube at 960 rcf for 3′ at 4 °C and remove any residual TE buffer.
8. Add 220 μL of elution buffer.
9. Elute at 65 °C for 1 h in the thermomixer at 200 rcf (1400 rpm).
10. Spin down beads at 16,000 rcf for 1′ at RT.
11. Remove 200 μL of supernatant and transfer to a new screw-cap tube. Reverse cross-link of this IP DNA by incubating at 65 °C overnight.
12. Thaw 20 μL of input sample reserved after sonication (**step 2** in Subheading 3.1.4), add 180 μL of elution buffer and mix. Reverse cross-link of this input DNA by incubating at 65 °C overnight.

3.1.6 DNA Precipitation

1. Add 200 µL of TE to each tube of IP and input DNA to dilute SDS in elution buffer.
2. Add 8 µL of 10 mg/mL RNaseA (0.2 ug/ml final concentration).
3. Mix and incubate at 37 °C for 1 h.
4. Add 4 µL of 20 mg/mL proteinase K (0.2 µg/mL final concentration).
5. Mix and incubate at 55 °C for 1 h.
6. Add 400 µL phenol:chloroform:isoamyl alcohol, vortex extensively (~1′), and spin at 16,000 rcf at RT for 5′.
7. Transfer 380 µL of aqueous layer to a new centrifuge tube containing 16 µL of 5 M NaCl (200 mM final concentration) and 1.5 µL of 20 ug/µL glycogen (30 µg total).
8. Add 1140 µL EtOH. Incubate for 1 h at −80 °C.
9. Spin at 20,000 rcf for 30′ at 4 °C to pellet DNA. Wash pellets with 1 mL of 70% EtOH.
10. Dry pellets and resuspend each in 30 µL of ddH_2O.
11. Dilute input ~25 times, and run analytical qPCR reaction, using 1–2 µL of ChIP'd DNA and 1–2 µL of diluted input DNA (*see* **Note 6**).

3.2 Co-immunoprecipitation: Co-IP

This is a rapid Co-IP protocol to test if pairs of nuclear proteins can interact in chick embryo cells (Fig. 2). The proteins of interest are cloned in separate PCI expression vectors (PCI-H2B-RFP [6] or similar), with AVI or Flag tags, respectively (we usually add the tags in the C-terminus of the protein, followed by a GSG linker). The AVI tag is added to the bait protein and the Flag tag is linked to the putative cofactor. A third vector, driving expression of nuclear BirA enzyme, is co-transfected in the embryos [7] to biotinylate the AVI tag of the bait protein. Following electroporation [5] (*see* **Note 7**), embryos will be cultured until the desired stage (HH9–10), dissected, and processed for extraction of nuclear proteins. Streptavidin beads (Dynabeads™ MyOne™ Streptavidin T1) are used for the immunoprecipitation of the bait protein. We then employ western blot to test the interaction between the bait and the putative cofactor. We often use this protocol in conjunction with proximity ligation assays (PLA) [8], which can be used to confirm that the interaction takes place in vivo under endogenous conditions.

3.2.1 Embryo Extract

1. Dissect embryos using filter paper and transfer to Ringer's solution (*see* **Note 8**).
2. Cut embryos out of filter paper, remove as much of membrane as possible, and transfer to a 1.7 mL tube (*see* **Note 3**). Perform 1 extract per sample.

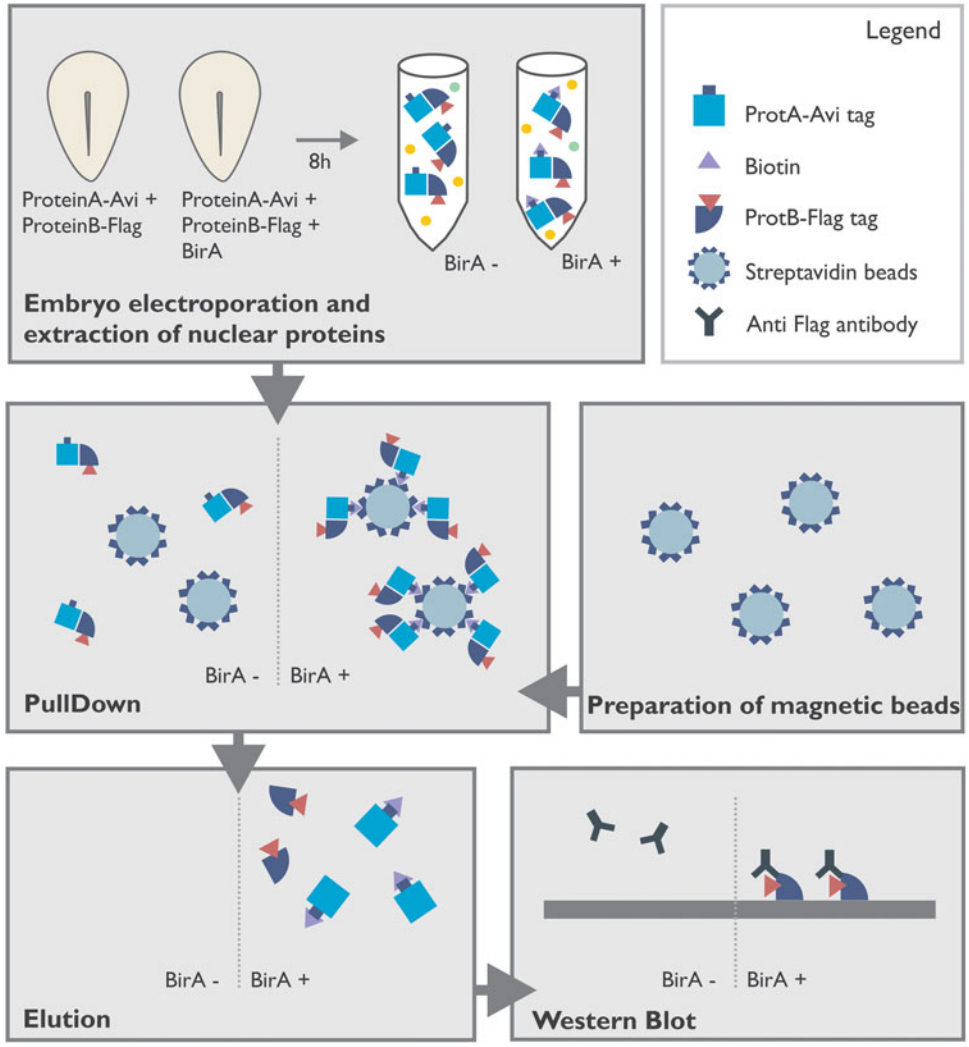

Fig. 2 Experimental design of a Co-IP experiment

3. Wash once with PBS.
4. Spin down at ~300 rcf, being careful not to lyse the embryos. Remove PBS.
5. Add 100 μL of hypotonic buffer to the embryos. Leave on ice for 15′ (*see* **Note 4**).
6. Dissociate the cells by aspirating and releasing the solution (~7 times) using a 1 mL syringe and a 27.5 gauge needle.
7. Incubate on ice for 15′.
8. Centrifuge at top speed for 2′ at 4 °C to spin out the lysate. The cytoplasmic protein is in the supernatant. If you wish to keep it, transfer the supernatant to a fresh tube and snap freeze in liquid

nitrogen. If you are only interested in the nuclear proteins, it can be discarded.

9. Resuspend the pellet in 10 μL of IP extraction buffer.
10. Rotate at 4 °C for 30′ to extract nuclear proteins.
11. Centrifuge at top speed for 2′ at 4 °C to spin out the debris.
12. Collect supernatant (nuclear extract) and transfer to a fresh tube.
13. Dilute 10 μL nuclear extract in 390 μL modified RIPA buffer for a final volume of 400 μL (*see* **Note 9**).

3.2.2 Preparation of Magnetic Beads

1. Gently resuspend the MyONE Dynabeads.
2. Transfer the 50 μL of magnetic beads to a microcentrifuge tube.
3. Using a magnetic stand to separate the bead, wash beads three times in PBS + 1% BSA FV (filtered).
4. Block beads at 4 °C in PBS + 1% BSA FV for 1 h on rotisserie.
5. Separate on a magnetic stand and remove supernatant.

3.2.3 Pull Down

1. Save an aliquot of 40 μL of the diluted nuclear extract from **step 13** in Subheading 3.2.1 and keep at −80 °C to be used as input sample.
2. Add the remaining diluted nuclear extract to the magnetic beads from **step 5** in Subheading 3.2.2.
3. Incubate for 2 h on rotisserie at 4 °C.
4. Let tubes sit for at least 3′ in magnetic stand to collect the beads. Remove and discard supernatant.
5. Add 1 mL of modified RIPA buffer to each tube. Remove tubes from magnetic stand and tap the tube gently to resuspend beads. Leave tubes on the nutator for 1–2′ for more efficient wash. Replace tubes in magnetic stand to collect beads. Remove supernatant.
6. Repeat this wash four more times.
7. After the last wash, change to chromatin/bead suspension to a new chilled microcentrifuge tube. This step largely reduces the nonspecific background.
8. Separate the beads using the magnetic stand and remove the supernatant as previously.
9. Resuspend each sample with:

   Sample reducing agent (10×)—5 μL.

   LDS sample buffer (4×)—12.5 μL.

   Modified RIPA buffer—32.5 μL.

10. Heat samples at 80 °C, for 15′ at 1400 rpm.
11. Separate the beads using the magnetic stand. Transfer the supernatant to a fresh tube. Store at −80°C.
12. To each aliquot of input (**step 1** in Subheading 3.2.3), add:

    Sample reducing agent (10×)—5 μL.

    LDS sample buffer (4×)—12.5 μL.
13. Heat samples at 80 °C, for 15′ at 1400 rpm. Store at −80 °C.
14. Run samples in a protein gel and perform western blot using an anti-Flag antibody. Use an anti-Flag antibody conjugated with HRP to speed up the analysis and minimize background.

4 Notes

1. PMSF stock solution is dissolved in ethanol as the half-life in water is 30′ in water.
2. The protease inhibitor stock solution is stable for 12 weeks at −15 to −25 °C.
3. For all steps in the procedure use low-binding safe-seal tubes. The use of low-retention pipette tips is preferred for accurate solution transfers. Also, the use of P1000 REACH tips helps to not disturb the beads during washes.
4. All steps prior to DNA elution should be done on ice to avoid protein degradation.
5. Use a ChIP-grade rabbit or mouse protein-specific (transcription factor) antibody. Also, use a ChIP-grade rabbit or mouse IGG as negative control.
6. Samples should be kept at −80 °C and analyzed within 2 weeks as the low-DNA-content samples degrade quickly. Enrichment of target region should be presented as percentage of input or as fold over the IGG DNA pull down.
7. For ex ovo electroporation [5], we recommend using the following concentrations of the PCI-H2B-RFP vectors: (a) BirA positive sample: Avi-tag plasmid (500 ng/μL) + Flag-tag plasmid (500 ng/μL) + BirA plasmid (500 ng/μL); (b) BirA negative sample: Avi-tag plasmid (500 ng/μL) + Flag-tag plasmid (500 ng/μL) + empty PCI-H2B-RFP (500 ng/μL).
8. Each aliquot of nuclear extract is prepared from at least 5–10 whole embryos (HH9–10) overexpressing the proteins of interest fused to Avi or Flag tags.
9. We generally use 10–20 μg of nuclear extract per IP.

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Chapter 8

Experimental Manipulation of Ploidy in Zebrafish Embryos and Its Application in Genetic Screens

Triveni Menon and Sreelaja Nair

Abstract

Metazoan animals are typically diploid, possessing two sets of a chromosome in the somatic cells of an organism. In naturally diploid species, alteration from the endogenous diploid state is usually embryonic lethal. However, the ability to experimentally manipulate ploidy of animal embryos has fundamental as well as applied biology advantages. In this chapter we describe experimental procedures to convert normally diploid zebrafish embryos into haploid or tetraploid states. We also describe methodologies to verify the ploidy of embryos and the utility of ploidy manipulation in expediting the isolation of mutations using both forward and reverse genetic strategies in zebrafish.

Key words Zebrafish, Mutagenesis, Ploidy, Haploid, Tetraploid, Gynogenesis, Heat shock, Genetic screen

1 Introduction

The original version of Hugo De Vries' mutation theory of evolution was based on his observation that species of the evening primrose *Oenothera lamarckiana* (*O. lamarckiana*) often displayed dramatic changes in physical form. De Vries ascribed these changes to mutations in *O. lamarckiana* genes [1]. However, it is now known that what De Vries interpreted as mutations was in fact the duplication of all 14 chromosomes of *O. lamarckiana* resulting in a new polyploid species, *O. gigas* with 28 chromosomes [1]. In the century that followed De Vries' original observation, the remarkable ability of plants to tolerate polyploidy has been a horticultural and agricultural boon to mankind, leading to production of seedless fruits, larger size produce, and drought-resistant crops. In stark contrast, the diploid state of chromosomes is an obligate requirement for embryonic survival in metazoans. The fundamental biological principles underlying this phenomenon remain unknown and are not pertinent for this chapter. However, the ability to manipulate the endogenous ploidy of embryos can be an

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_8,

experimental advantage in model organisms typically used to understand how embryonic development occurs in metazoans.

A vertebrate species in which experimental manipulation of endogenous ploidy has instantaneous genetic advantages is the freshwater minnow, *Danio rerio*, commonly known as zebrafish. With ~70% of human proteins having an ortholog in zebrafish, this vertebrate species has emerged as a dependable developmental and biomedical research model. Additionally, zebrafish are amenable to unbiased forward as well as targeted reverse genetics approaches. Short generation time, large clutches of embryos from a mating pair, optical transparency of embryos for live imaging, ease of maintenance, and lower animal husbandry costs also add to the advantages of zebrafish, an attractive research model system. Herein we describe experimental procedures to generate haploid and tetraploid zebrafish embryos and methodologies to validate the altered ploidy state. We also describe procedures to combine the two ploidy manipulation paradigms with a traditional forward genetic screen or a CRISPR-Cas9-based reverse genetic strategy for expedited identification of recessive zygotic and maternal effect mutations in diploidized zebrafish haploids.

1.1 Ploidy Manipulation in Zebrafish

In animals, haploid individuals can arise by three means: parthenogenesis, androgenesis (embryonic development with only paternal DNA), and gynogenesis (embryonic development with only maternal DNA). Parthenogenesis is a natural form of asexual reproduction wherein the haploid egg develops either without fertilization or without DNA contribution from the sperm after fertilization, and is common in invertebrates such as insects, nematodes, and reptiles [2]. Naturally occurring parthenogenesis is rare in vertebrates but does occur in some species of frogs and fish [2]. In species where parthenogenesis does not occur naturally, haploidy can be induced by a variety of experimental manipulations. In mammals, eggs can be chemically treated to generate gynogenotes or the sperm nucleus is transferred into an enucleated egg to generate androgenotes [3–5]. Haploidy can also be generated by eliminating either the female pronucleus (androgenesis) or the male pronucleus (gynogenesis), for example, by using maternal or paternal effect mutations in *D. melanogaster* [6]. In the absence of a genetic methodology, vertebrate experimental biologists typically irradiate the egg or the sperm to destroy the female or male pronucleus, respectively. The irradiated gametes are then used in in vitro fertilizations with normal sperm or eggs to produce androgenetic and gynogenetic frog, medaka, and zebrafish embryos [7–10]. Though such experimentally generated haploids do not survive to adults, haploid vertebrate embryos are of particular importance in establishing haploid embryonic stem cell lines for medical genetics studies and for zygotic recessive genetic screens such as those done in zebrafish [4, 11–13].

A major advantage of the experimental tractability of zebrafish as a model system lies in the ease with which gametes can be obtained, manipulated, and in vitro fertilized to generate embryos. In addition to the ease with which zebrafish haploids can be generated, preventing the first cell division by applying a late hydrostatic pressure or transient heat shock results in production of zebrafish tetraploids [10, 14]. During this process, the duplicated DNA fails to segregate into daughter cells due to failed cytokinesis and undergoes another round of DNA replication, thereby doubling genome content in the embryos. When this methodology is used on haploid zebrafish, the embryos become diploids, which is particularly useful because unlike the haploids, diploidized zebrafish embryos are viable and grow to be fertile, clonal adults. Relatively, the transient heat paradigm is technically simpler requiring only a water bath than the transient hydrostatic pressure paradigm, which requires a specific instrument such as a French or hydraulic pressure cell.

In this chapter, in addition to zebrafish gynogenetic haploid production (Fig. 1b), we also describe an optimized methodology of using transient heat as a genome-doubling strategy for zebrafish embryos (Fig. 2). We also describe three methodologies (metaphase chromosome spreads, fluorescence-assisted cell sorting (FACS), and parental trait assessment), any of which can be used to verify the ploidy of zebrafish embryos.

1.2 Expedited Identification of Recessive Mutations Using Gynogenic Diploids

Forward genetic screens in zebrafish have used the alkylating chemical mutagen N-ethyl-N-nitrosourea (ENU) to cause point mutations in the premeiotic adult male germ line. Mutagenized males are bred with normal females and the F1 and F2 generations of heterozygotes are sequentially inbred to identify zygotic recessive mutations in the F3 embryos [15–19]. For identification of maternal recessive mutations, the F3 embryos are raised to adults and the F4 embryos from the F3 adult females are screened for phenotypes, which takes an additional ~3 months [18, 20–23]. The ability to generate zebrafish haploids and subsequent diploidization of the haploids by transient hydrostatic pressure or heat provides an unmatched experimental ease in homozygosing genetic loci one generation early, which is also a time- and cost-saver advantage for the experimenter [18, 20–23]. For genetic screens, the diploidization process using late hydrostatic pressure or transient heat produces homozygotes at a predictable 50% frequency in the clutch of embryos from a heterozygous parent [10, 23].

In this chapter, we also describe the combined use of gynogenetic haploid and subsequent genome doubling by transient heat shock for the production of gynogenetic diploid embryos (Figs. 1 and 3). Such gynogenetic diploids can be screened for zygotic

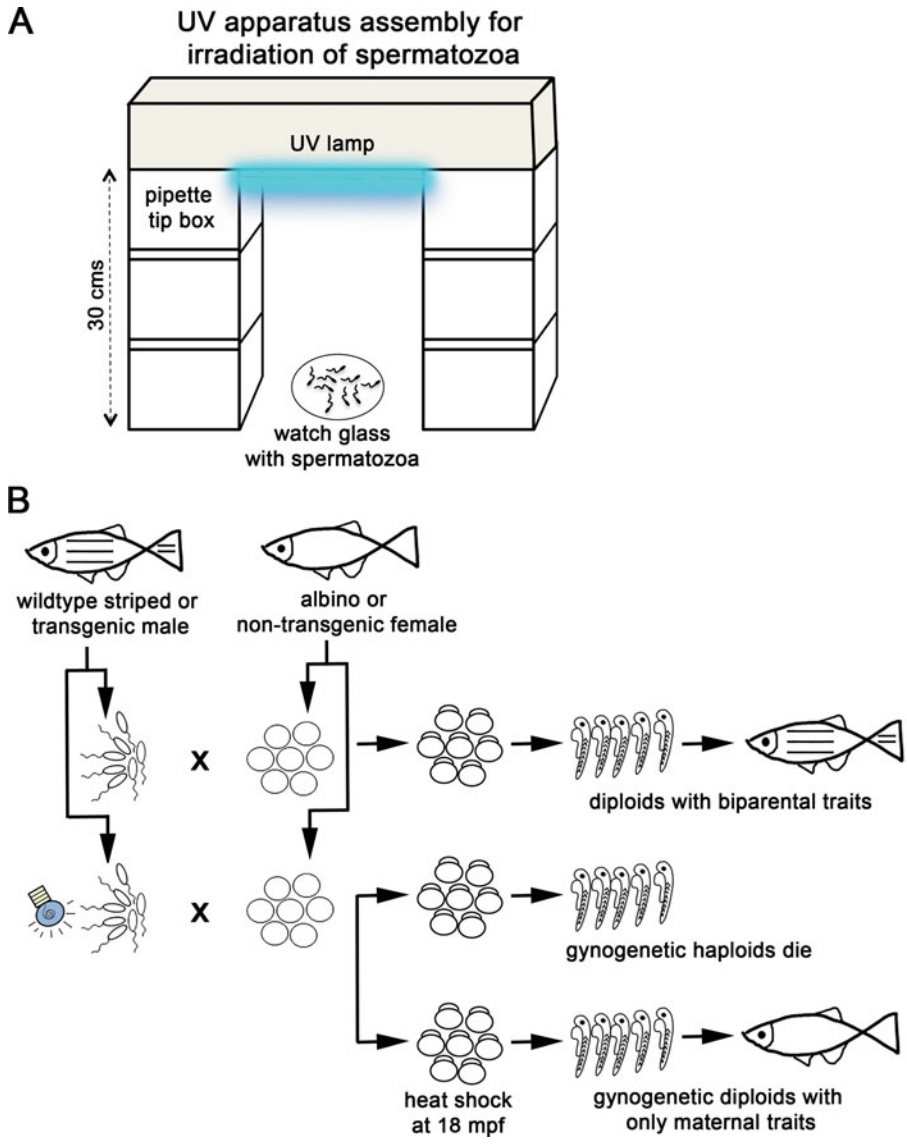

Fig. 1 (**a**) Schematic depiction of an UV irradiation setup. A handheld UV lamp (example: Genei, 6 W) is held at a distance of 30 cms by placing it atop a stack of empty pipette tip boxes. The watch glass containing spermatozoa supernatant is placed in the center of the setup, on the bench. (**b**) IVF using spermatozoa and eggs. Typically, IVF results in diploid progeny that inherits traits from both parents. Inheritance of paternal specific traits can be assessed by using spermatozoa from males harboring traits that are missing in the females (such as pigmentation or a transgene insertion such as *Tg(cmlc2:EGFP)*). Using UV-irradiated spermatozoa from males carrying a paternal trait ensures that if the sperm nucleus has been fully destroyed, the resultant progeny will not inherit the paternal trait, which can be assayed for (pigmentation or transgene presence in progeny). Embryos obtained using UV-irradiated spermatozoa and eggs are gynogenic haploids carrying only maternal traits, which never survive to adulthood. However, if such embryos are transiently heat shocked, they become gynogenic diploids carrying only maternal traits and grow to adulthood

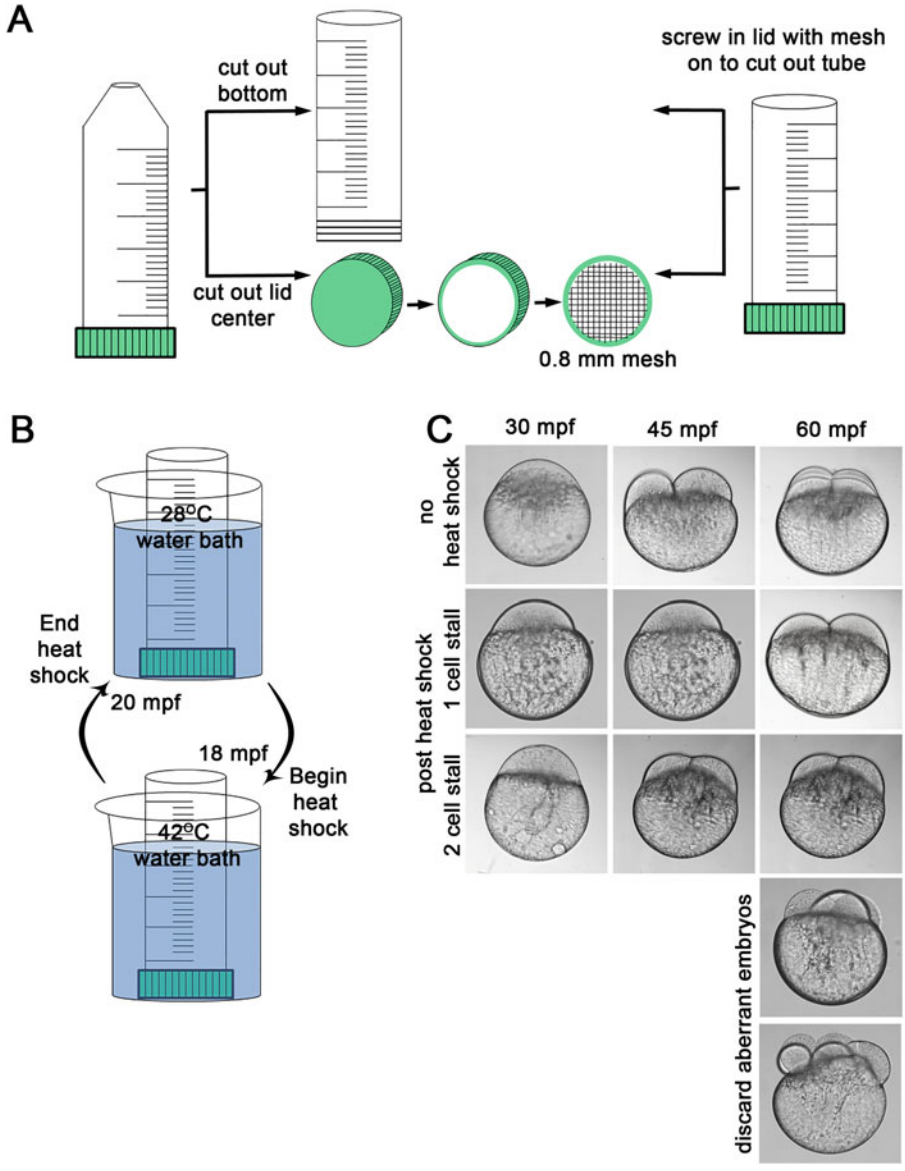

Fig. 2 (**a**) The conical bottom of a 50 mL tube is cut to obtain an open tube. Cut out the center of the screw-cap lid and place a piece of 0.8 mm mesh. Screw the lid with the mesh on to the tube to form an immersible mesh-bottom tube. (**b**) Keep two water baths, one at 42 °C and one at 28 °C, to heat shock and quench the heat shock instantly. Transfer embryos between the two water baths using the mesh-bottom tube. (**c**) Typical results of the transient heat shock done at 18 mpf. One-cell and two-cell stall embryos are behind control embryos by one cell cycle and are true tetraploids. Aberrant cleaving embryos are technical casualties of the experiment, which should be discarded

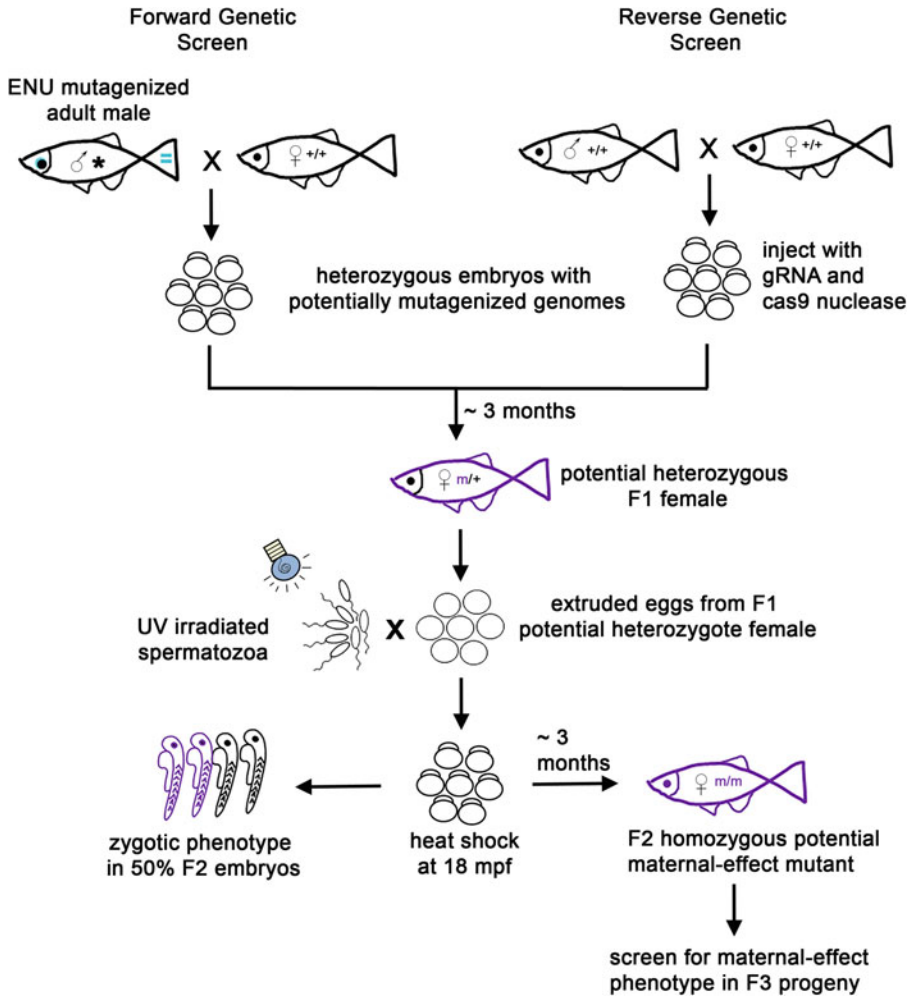

Fig. 3 Expedited strategy for screening zygotic and maternal effect mutations from both forward and reverse genetic screens in gynogenic diploids. Progeny from ENU mutagenized males or one-cell embryos injected with guide RNA and cas9 nuclease are raised to F1 adults. These F1 adults are carriers of potential mutations and can be screened at 3 months. Eggs from F1 females can be fertilized with UV-irradiated spermatozoa to generate haploids, which can be diploidized by a transient heat shock to produce F2 gynogenic diploids. If the F1 female is a carrier for a zygotic recessive mutation, 50% of the gynogenic diploids will manifest a zygotic phenotype. If the F1 female is a carrier for a maternal effect recessive mutation, 50% of the F2 adults will be homozygous for the mutation. 100% of F3 embryos obtained from a homozygous F2 female will manifest the maternal effect phenotype

phenotypes or raised to adults and screened for maternal effect phenotypes in the next generation (Fig. 3). The use of gynogenetic zebrafish diploids in a mutation screen accelerates the identification of recessive mutant loci by one entire generation.

2 Materials

2.1 Ploidy Manipulation in Zebrafish

2.1.1 Generation of Gynogenetic Haploids

1. UV-irradiated spermatozoa solution (Subheading 3.4.3).
2. Freshly extruded eggs.
3. Embryo medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% methylene blue.

2.1.2 Generation of Tetraploids

1. Spermatozoa supernatant (Subheading 3.4.1).
2. Freshly extruded eggs (Subheading 3.4.2).
3. Embryo medium.
4. 50 mL Mesh-bottom tubes (made by cutting away the conical bottom of a 50 mL tube and making a hole in the lid; screwing on the lid with a mesh in place allows the use of the tube as an immersible container).
5. Water bath at 28 °C containing a 250 mL beaker with embryo medium.
6. Water bath at 42 °C containing a 250 mL beaker with embryo medium.

2.1.3 Generation of Gynogenetic Diploids

1. UV-irradiated spermatozoa (Subheading 3.4.3).
2. Freshly extruded eggs (Subheading 3.4.2).
3. Embryo medium.
4. 50 mL Mesh-bottom tubes.
5. Water bath at 28 °C containing a 250 mL beaker with embryo medium.
6. Water bath at 42 °C containing a 250 mL beaker with embryo medium.

2.2 Assessing Ploidy of Zebrafish Embryos

2.2.1 Assessing Ploidy by Metaphase Chromosome Spreads

1. Fine dissection forceps (#55).
2. Glass petri dishes.
3. Watch glass.
4. Multi-well glass dish.
5. Colchicine solution: 4 mg/mL Colchicine in embryo medium without methylene blue.
6. 1.1% Sodium citrate.
7. 1–10 μL PCR micropipettes.
8. 50% Acetic acid.
9. Freshly made 3:1 methanol:acetic acid.
10. Metal sheet on a heat block held at 65 °C to preheat slides.
11. DNA stain such as DAPI.
12. Tissue wipes.

2.2.2 Assessing Ploidy by Fluorescence-Assisted Cell Sorting (FACS)

1. Fine dissection forceps (#55).
2. Fish Ringer's solution without Ca^{2+}: 55 mM NaCl, 1.8 mM KCl, 12.5 mM $NaHCO_3$.
3. 1× Phosphate-buffered saline.
4. Trypsin solution: 0.2% Trypsin, 0.14 M NaCl, 5 mM KCl, 5 mM D-glucose, 7 mM $NaHCO_3$, and 0.7 mM EDTA pH 7.2.
5. 70% Ethanol.
6. Benchtop centrifuge.
7. 40 μg/mL Propidium iodide in distilled water.
8. 10 μg/mL RNase.
9. Flow cytometer.

2.2.3 Assessing Ploidy by Assaying for Paternal Traits in Gynogenic Diploids

1. UV-irradiated spermatozoa from wild-type striped males or from males carrying a transgenic insertion such as *Tg(cmlc2:EGFP)*.
2. Freshly extruded eggs (Subheading 3.4.2).
3. Embryo medium.
4. Stereomicroscope with camera.

2.3 Expedited Identification of Recessive Mutations Using Gynogenic Diploids

1. Female F1 progeny from ENU mutagenesis or CRISPR guide RNA-injected fish.
2. Freshly extruded eggs (Subheading 3.4.2) from the above F1 females.
3. UV-irradiated spermatozoa (Subheading 3.4.3).
4. Embryo medium.
5. 50 mL Mesh-bottom tubes.
6. Water bath at 28 °C containing a 250 mL beaker with embryo medium.
7. Water bath at 42 °C containing a 250 mL beaker with embryo medium.

2.4 Additional Experimental Procedures

2.4.1 Extraction of Spermatozoa from Adult Testes

1. Adult male zebrafish.
2. MESAB stock: 0.4% Ethyl-m-aminobenzoate methanesulfonate in 1X phosphate-buffered saline, pH 7.4.
3. MESAB working solution: 0.05% Ethyl-m-aminobenzoate methanesulfonate in fish water. Combine 30 mL MESAB stock and 200 mL of fish facility water.
4. Hanks' solution 1: 8 gm NaCl + 0.4 gm KCl in 100 mL distilled water. Store at 4 °C.

5. Hanks' solution 2: 0.358 gm Na_2HPO_4anhydrous + 0.6 gm KH_2PO_4 in 100 mL distilled water. Store at 4 °C.
6. Hanks' solution 4: 0.72 gm $CaCl_2$ in 50 mL distilled water. Store at 4 °C.
7. Hanks' solution 5: 1.23 gm $MgSO_4{\cdot}7H_2O$ in 50 mL distilled water. Store at 4 °C.
8. Hanks' solution 6: 0.35 gm of $NaHCO_3$in 10 mL of distilled water, prepared fresh.
9. Hanks' premix: Mix Hanks' solutions in this order: 10 mL solution 1 + 1 mL solution 2 + 1 mL solution 4 + 86 mL distilled water and 1 mL of solution 5. Store at 4 °C.
10. Hanks' buffer: 900 μL Hanks' premix +10 μL Hanks' solution 6, prepare fresh.
11. Dissection scissors.
12. Dissection forceps (#3 or #5).
13. An incident light source.
14. A spoon and a net.

2.4.2 Extrusion of Eggs from Adult Females

1. Egg-laying primed adult females that were set up in pairwise matings the previous night.
2. MESAB stock: 0.4% Ethyl-m-aminobenzoate methanesulfonate in 1× phosphate-buffered saline, pH 7.4.
3. MESAB working solution (30 mL stock +200 mL of fish facility water).
4. A net.
5. Flat-end spatula.

2.4.3 UV-Inactivated Spermatozoa Solution

1. Spermatozoa supernatant.
2. Medium-sized watch glass.
3. UV lamp (6 W).

2.4.4 In Vitro Fertilization of Eggs with Spermatozoa Solution

1. Spermatozoa supernatant.
2. Freshly extruded eggs.
3. Dry petri dishes.
4. Embryo medium.

3 Methods

3.1 Ploidy Manipulation in Zebrafish

3.1.1 Generation of Gynogenetic Haploids

1. Set up pair matings the previous night of the experiment. When the lights turn on the next morning, watch the pairs and separate from the male the females that have just begun to lay eggs.
2. Extrude eggs from such egg-laying primed females and in vitro fertilize the eggs with UV-inactivated spermatozoa (Fig. 1a, use 200 μL spermatozoa solution for ~200 eggs).
3. At 40–45 min postfertilization (mpf), observe embryos under a transmitted light stereomicroscope and check for possible polyspermy (*see* **Note 1**).
4. Incubate embryos at 28 °C, and observe and document phenotypes.

3.1.2 Generation of Tetraploids

1. Set up pair matings the previous night of the experiment. When the lights turn on the next morning, watch the pairs and separate from the male the females that have just begun to lay eggs.
2. Extrude eggs from such egg-laying primed females and in vitro fertilize the eggs with spermatozoa supernatant (200 μL spermatozoa solution for ~200 eggs). Start a timer.
3. At 4 min postfertilization, transfer ~50 embryos to a 50 mL mesh-bottom tube and place the tube in a 28 °C embryo medium bath (Fig. 2a, b).
4. 10 s before 18 min postfertilization, transfer the mesh-bottom tube into a 42 °C embryo medium bath (*see* **Note 2**).
5. At 20 min postfertilization, quench the heat shock by transferring the mesh-bottom tube back into the 28 °C embryo medium bath.
6. Allow embryos to recover from the heat shock for 2 min in the 28 °C embryo medium bath and then transfer them into plastic petri dishes.
7. Monitor the embryos for the one-cell-cycle cytokinesis stall as an indicator of success for genome duplication (Fig. 2c; *see* **Notes 3–5**).
8. Transfer embryos that underwent a cytokinesis stall into a new petri dish and incubate at 28 °C. Observe and document phenotypes at the requisite developmental stages.

3.1.3 Generation of Gynogenetic Diploids

1. Set up pair matings the previous night of the experiment. When the lights turn on the next morning, watch the pairs and separate from the male the females that have just begun to lay eggs.

2. Extrude eggs from such egg-laying primed females and in vitro fertilize the eggs with UV-inactivated spermatozoa (Fig. 1a, use 200 μL spermatozoa solution for ~200 eggs). Start a timer.
3. At 4 min postfertilization, transfer ~50 embryos to a 50 mL mesh-bottom tube and place the tube in a 28 °C embryo medium bath (Fig. 2a, b).
4. 10 s before 18 min postfertilization, transfer the mesh-bottom tube into a 42 °C embryo medium bath (*see* **Note 2**).
5. At 20 min postfertilization, quench the heat shock by transferring the mesh-bottom tube back into the 28 °C embryo medium bath.
6. Allow embryos to recover from the heat shock for 2 min in the 28 °C embryo medium bath and then transfer them into plastic petri dishes.
7. Monitor the embryos for the one-cell-cycle cytokinesis stall as an indicator of success for genome duplication (Fig. 2c; *see* **Notes 3–5**).
8. Transfer embryos that underwent a cytokinesis stall into a new petri dish and incubate at 28 °C (*see* **Note 6**). Observe and document phenotypes at the requisite developmental stages.

3.2 Assessing the Ploidy of Zebrafish Embryos

3.2.1 Assessing Ploidy by Metaphase Chromosome Spreads

1. Generate diploid, tetraploid, and gynogenetic diploid embryos as described in Subheading 3.1.
2. Manually dechorionate ~10 24–30 hpf larvae using #55 fine forceps and transfer them to a 9-well glass plate.
3. Remove excess embryo medium and add 100 μL of embryo medium containing 4 mg/mL colchicine. Incubate the plate in the dark at 28 °C for 5 min.
4. Replace colchicine with 300 μL of fresh embryo medium containing 4 mg/mL colchicine. Incubate the plate in the dark at 28 °C for 90 min.
5. Discard colchicine and add 300–500 μL embryo medium without methylene blue to rinse embryos.
6. Discard embryo medium rinse, add 1 mL 1.1% sodium citrate solution, and start a timer. Puncture the yolk of the larvae using a pair of #55 fine forceps (*see* **Note 7**).
7. At the end of 8 min, transfer the 9-well glass plate onto a flat tray of ice and incubate on ice for an additional 8 min.
8. Transfer the embryos into 1 mL 3:1 solution of methanol: acetic acid and incubate at 4 °C overnight.
9. Next day, preheat glass slides for 45 min to 1 h at 65 °C.
10. Transfer embryos onto a clean watch glass and remove excess fixative by blotting. Add three drops of 50% acetic acid solution

onto the embryos and mince thoroughly using a pair of #55 fine forceps (*see* **Note 8**).

11. Triturate the embryos thoroughly using a 10 μL PCR micropipette to form a single-cell suspension.
12. Keep a Bunsen burner or ethanol-wick lamp ready. Using the 10 μL PCR micropipette drop a few drops of the cell suspension onto the preheated glass slides.
13. Before the suspension evaporates from the slide, flame the glass slide thrice to dry.
14. Dry the slides at 65 °C for 60 min on the metal sheet.
15. Stain the DNA using a DNA stain such as DAPI containing an antifade compound.
16. Scan the slides for metaphase spreads and image using an epifluorescence microscope or confocal microscope.
17. Count the chromosomes manually using the multipoint tool in ImageJ or by using the analyze particle plug-in.

3.2.2 Assessing Ploidy by Fluorescence-Assisted Cell Sorting (FACS)

1. Generate diploid, tetraploid, and gynogenetic diploid embryos as described in Subheading 3.1.
2. Manually dechorionate ~50 24–30 hpf larvae using #55 fine forceps and transfer them into a 1.5 mL tubes using a glass Pasteur pipette.
3. Discard excess embryo medium and add 1 mL of Ringer's solution without Ca^{2+}.
4. Using a glass pipette, deyolk the embryos by repeated pipetting.
5. Centrifuge the tube at 1000 × *g* for 5 min and discard the supernatant which now contains the yolk.
6. Add 1 mL of ice-cold trypsin solution and triturate using a 10 μL PCR micropipette to make a single-cell suspension.
7. Pellet the cells by centrifuging at 1000 × *g* for 7 min at room temperature.
8. Discard the supernatant and resuspend the cell pellet in 200 μL of sterile 1 × PBS.
9. Add 1 mL 70% ethanol and incubate at 4 °C overnight to fix cells. This cell suspension can now be stored at 4 °C and for a few months at −20 °C.
10. Centrifuge the overnight-fixed cell suspension at 1000 × *g* for 10 min at room temperature to pellet the cells.
11. Discard the supernatant and resuspend the cell pellet in 200 μL of propidium iodide solution (40 μg/mL) and RNase solution (10 μg/mL).

12. Incubate the tubes in the dark at 28 °C for 30 min.
13. Add nuclease-free water to 1 mL and load the entire sample onto a flow cytometer for sorting.

3.2.3 Assessing Ploidy by Assaying for Paternal Traits in Gynogenic Diploids

1. Prepare spermatozoa solution from transgenic male fish such as *Tg(cmlc2:EGFP)* (*see* **Note 9**).
2. Irradiate 200 μL of spermatozoa solution using 254 nm UV light for 90 s and place on ice (*see* Fig. 1).
3. Extrude eggs from egg-laying primed female (nonpigmented, non-transgenic, F1 progeny from ENU mutagenesis or CRISPR guide RNA injected fish) and in vitro fertilize the eggs with UV-inactivated spermatozoa (200 μL spermatozoa solution for ~200 eggs). Start a timer.
4. At 4 min postfertilization, transfer ~50 embryos to a 50 mL mesh-bottom tube and place the tube in a 28 °C embryo medium bath (*see* Fig. 2).
5. 10 s before 18 min postfertilization, transfer the mesh-bottom tube into a 42 °C embryo medium bath.
6. At 20 min postfertilization, quench the heat shock by transferring the mesh-bottom tube back into the 28 °C embryo medium bath.
7. Allow embryos to recover from the heat shock for 2 min in the 28 °C embryo medium bath and then transfer them into plastic petri dishes.
8. Monitor the embryos for the one-cell-cycle cytokinesis stall as an indicator of success for genome duplication. Embryos that will become gynogenic diploids are one celled or two celled when non-heat-shocked embryos are two celled or four celled, respectively (*see* Fig. 2).
9. Transfer embryos that underwent a cytokinesis stall into a new petri dish and incubate at 28 °C.
10. Verify the absence of paternal traits (pigmentation, transgene presence, etc.) in such gynogenic diploid embryos at 24 or 48 hpf.

3.3 Expedited Identification of Zygotic and Maternal Effect Recessive Mutations Using Gynogenetic Diploids

1. Set up female F1 progeny from ENU mutagenesis or CRISPR guide RNA-injected fish in pairwise matings with wild-type males on the previous night of the experiment (Fig. 3).
2. On the morning of the experiment prime the female F1 progeny by allowing for initiation of natural egg laying at the onset of the light cycle.
3. Separate the primed F1 females into a new tank and label each egg-laying primed female fish individually.

4. Prepare spermatozoa solution from transgenic male fish such as *Tg(cmlc2:EGFP)* (*see* **Note 9**).
5. Irradiate 200 μL of spermatozoa solution using 254 nm UV light for 90 s and place on ice.
6. Extrude eggs from an egg-laying primed F1 and in vitro fertilize the eggs with UV-inactivated spermatozoa (200 μL spermatozoa solution for ~200 eggs). Start a timer.
7. At 4 min postfertilization, transfer ~50 embryos to a 50 mL mesh-bottom tube and place the tube in a 28 °C embryo medium bath.
8. 10 s before 18 min postfertilization, transfer the mesh-bottom tube into a 42 °C embryo medium bath.
9. At 20 min postfertilization, quench the heat shock by transferring the mesh-bottom tube back into the 28 °C embryo medium bath.
10. Allow embryos to recover from the heat shock for 2 min in the 28 °C embryo medium bath and then transfer them into plastic petri dishes.
11. Monitor the embryos for the one-cell-cycle cytokinesis stall as an indicator of success for genome duplication. Embryos that will become gynogenic diploids are one celled or two celled when non-heat-shocked embryos are two celled or four celled, respectively.
12. Transfer embryos that underwent a cytokinesis stall into a new petri dish and incubate at 28 °C.
13. Assess such gynogenic diploids for zygotic recessive embryonic phenotypes of interest and check for the absence of cardiac fluorescence at 24 or 48 h postfertilization to verify the ploidy as gynogenic diploid.
14. Embryos from female gynogenic diploid fish that survive to adulthood can be assayed for recessive maternal effect phenotypes.

3.4 Additional Experimental Procedures

3.4.1 Extraction of Spermatozoa from Adult Testes

1. Prepare 1 mL of Hanks' buffer in a 1.5 mL tube and chill on ice.
2. Euthanize an adult male zebrafish in MESAB and spoon out the fish onto some tissue paper.
3. Decapitate the fish on a plastic petri dish and make a longitudinal cut along the ventral side of the abdomen up to the gills.
4. Place the fish dorsal side down on a dissecting microscope with an incident light source and flay open the abdominal wall using a pair of #3 dissection forceps.
5. Remove the gut and swim bladder to expose the opaque, white, elongated testes along either sides of the abdomen.

6. Pick up the testes with the forceps and transfer into Hanks' buffer on ice.
7. Using a 200 μL pipette triturate the tissue by repeated pipetting. Allow the solution to settle on ice.
8. Supernatant contains spermatozoa and can be used for up to 2 h postdissection when kept on ice.

3.4.2 Extrusion of Eggs from Adult Females

1. Set up adult fish in pair matings the previous night of the experiment.
2. On the morning of the experiment, at the onset of the light cycle, monitor the pairs for egg-laying behavior.
3. As soon as egg laying initiates, separate the females into a new tank.
4. Anesthetize the egg-laying primed female in MESAB working solution and spoon out the female onto a wad of paper towel.
5. Gently pat dry the anal fin and gently press the abdomen using a flat-end spatula.
6. Move the eggs away from the body of the fish and transfer the fish back into fish water for revival.
7. Use the extruded eggs for in vitro fertilizations.
8. Rest the female fish for about 7–10 days before use for natural pair matings and for 15 days before another round of egg extrusion.

3.4.3 UV-Inactivated Spermatozoa Solution

1. Collect spermatozoa from adult male zebrafish as described in Subheading 3.4.1.
2. Transfer 100–200 μL of the spermatozoa supernatant onto a clean watch glass.
3. Irradiate the spermatozoa solution using 254 nm UV light from a 6 W UV lamp at a distance of 30 cm for 90 s with intermittent swirling every 30 s (*see* **Notes 10** and **11**).
4. Transfer the irradiated spermatozoa solution into a clean, pre-chilled 1.5 mL tube and place the tube on ice (*see* **Note 11**).
5. Spermatozoa solution can be used for up to 2 h postdissection when kept on ice.

3.4.4 In Vitro Fertilization of Eggs with Spermatozoa Solution

1. Add 100 μL of spermatozoa solution to a pool of ~200 freshly extruded eggs on clean petri dish and mix thoroughly by gentle swirling of the plate.
2. Activate the eggs by adding 500 μL of embryo medium and mix well by gentle swirling. Start a timer.
3. After 1 min flood the plate with embryo medium and allow the embryos to develop at 28 °C.

4 Notes

1. Eggs fertilized by a single spermatozoon (monospermy) inherit a pair of centrioles, which are required for postfertilization cytokinesis in the zygote. Eggs fertilized by multiple spermatozoa (polyspermy) inherit proportionately excess centrioles, which trigger multipolar mitotic spindles in the zygote. Such polyspermic embryos become multicellular (typically four-celled), after the first cell cycle, instead of becoming two-cell embryos. These can be easily identified at 45 min postfertilization and should be discarded.
2. Tetraploid embryos can be obtained by performing a transient heat stress during the first zygotic mitosis between 12 min postfertilization and 26 min postfertilization. However, transient heat shock administered at 18 min postfertilization, when the pronuclear congression has concluded but the first mitosis is yet to initiate, maximizes the survival of tetraploids to 5 days postfertilization [24]. Gynogenetic diploids thus generated also survive better facilitating the use of the transient heat-shock paradigm for expedited identification of mutations in screens.
3. Heat-shocked embryos must be observed carefully for cytokinesis stalls. Tetraploid embryos obtained by heat shock typically undergo one of the two kinds of cytokinesis stalls: they either remain as one-cell embryos when non-heat-shocked embryos become two celled or will remain as two-cell embryos when non-heat-shocked embryos become four-celled.
4. The clutch of heat-shocked embryos may also include embryos that do not manifest either types of cytokinesis stalls. These should be discarded.
5. The clutch of heat-shocked embryos may also include embryos that undergo asymmetric cleavages. These should be discarded.
6. Because gynogenetic diploids are generated by heat shocking gynogenetic haploid embryos, the combined effects of the haploid syndrome and the heat shock may compromise yield of such embryos. To ensure maximal survival and healthy conditions, split the clutch at 24 h postfertilization into petri dishes, each containing no more than 30 embryos. Embryo medium should be replaced daily and dead embryos discarded.
7. All larvae should get deyolked completely. Excess yolk prevents the single-cell suspension from spreading properly on the surface of the glass coverslip. It is advisable to start with a small number of embryos (~10) so that all embryos can be deyolked in a span of 8 min.

8. Overnight-fixed larvae should be minced thoroughly to obtain a single-cell suspension. Triturate repeatedly with the PCR micropipette (10 μL) to homogenize the embryos completely. Any leftover tissue interferes with effective spreading of the single-cell suspension on the glass coverslip. If any larval tissue remains after homogenization, remove with a pair of fine forceps before spreading the suspension.
9. It is easier to use wild-type pigmented male to generate the UV-irradiated spermatozoa solution for verification of haploidy and subsequent diploidization in gynogenic diploids, if the eggs are from albino females. However, in a mutation screen it is unlikely that the mutagenesis will be performed in an albino strain. Thus to assess the lack of genetic contribution from the male in haploids or gynogenic diploids, a transgenic male such as *Tg(cmlc2:EGFP)* can be used to generate UV-irradiated spermatozoa.
10. A range of UV dosage between 30 and 150 s (6–30 mJ/cm^2) can be used for the generation of gynogenetic haploids. A 90-s (18 mJ/cm^2) UV exposure minimizes the severity of the haploid syndrome and decreases lethality of gynogenetic haploids and diploids.
11. Efficient irradiation of spermatozoa solution can be achieved by swirling the spermatozoa solution intermittently during irradiation using a 200 μL pipette tip. Upon completion of irradiation, discard the tip and use a fresh pipette tip to transfer the irradiated spermatozoa solution onto ice. Using the pipette tip used for swirling may contaminate the irradiated spermatozoa solution with nonirradiated or partially irradiated spermatozoa, thereby compromising generation of true haploids.

Acknowledgments

Our research is funded by a Wellcome Trust/Department of Biotechnology India Alliance Intermediate Fellowship (IA/I/13/2/501042) to SN and by the Tata Institute of Fundamental Research, Mumbai.

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Chapter 9

RNA Tomography for Spatially Resolved Transcriptomics (Tomo-Seq)

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Abstract

Embryonic development is heavily dependent on temporally and spatially restricted gene expression. Spatially resolved measurements of gene expression are therefore crucial for identifying novel regulators and the understanding of their function. However, in situ methods do not resolve global gene expression, and sequencing-based methods usually do not provide spatial information. Here, we describe tomo-seq, a method that combines classical histological sectioning of embryos or tissues with a highly sensitive RNA-sequencing technique. Application of tomo-seq to zebrafish embryos allows reconstructing the spatial gene expression of thousands of genes.

Key words mRNA sequencing, Local gene expression, Spatial transcriptomics, Developmental patterning, Gene expression mapping, Morphogen gradients

1 Introduction

Establishment of spatially restricted gene expression patterns is a key process in the development of tissues and organs. So far the gold standard tool to visualize RNA localization has been whole-mount or section in situ hybridization [1], since the protocol and the assessment of the results are relatively simple. However, typically only one or a few genes can be analyzed per sample and experiment. Recently, in situ sequencing methods like MerFISH [2] and SeqFISH [3] have been developed to overcome this limitation: by sequential hybridization of short probes coupled to fluorescent dyes, some hundred genes and their localization can be assessed in a single-tissue section. While providing detailed spatial information, these protocols put high requirements on the equipment, and currently do not scale to the level of the whole transcriptome. Classical RNA-sequencing (RNA-seq), on the other hand, provides transcriptome-wide gene expression data, but spatial information is lost. However, novel approaches deduce spatial information from single-cell RNA-seq data by positioning cells

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_9,

according to the expression of known landmark genes [4–6] or detect spatial expression patterns in tissue sections directly using barcoded surfaces [7].

Here, we describe tomo-seq, a method for spatially resolved transcriptomics combining RNA barcoding techniques (as used in single-cell sequencing approaches) with classical tissue sectioning. Importantly, tomo-seq only requires standard lab equipment and access to a cryostat. The steps from the fresh sample to a sequenceable library can be accomplished in 5 to 7 days. Tomo-seq is a simple but powerful technique for assessing spatially restricted gene expression, and is particularly suitable for applications in developmental biology. The spatial expression of thousands of genes can be mapped simultaneously and consequently be used to describe animal development in an unbiased and systematic way.

2 Materials

2.1 Sample Preparation

1. Cryotome.
2. Tungsten needles.
3. Tissue Tek Cryomolds, 10 mm × 10 mm × 5 mm (Sakura).
4. Toothpicks (for transferring sections to tubes).
5. Jung tissue-freezing medium.
6. Blue polyacrylamide beads (Bio-Rad).
7. Nuclease-free water.
8. Dry ice.
9. TRIzol reagent (Ambion).
10. GlycoBlue Coprecipitant (Ambion).
11. ERCC RNA spike-in mix (Ambion).

2.2 RNA Extraction

1. Isopropanol.
2. Chloroform.
3. Ethanol.
4. Aluminum racks.

2.3 cDNA Synthesis and First Amplification Step

1. Primer sequences, as in CELseq2 [8]: Primers for first reverse transcription (barcoded, one per section):
   - Second reverse transcription primer
   - Library PCR primers with Illumina indices
2. Superscript II, first-strand buffer and 0.1 M DTT (Thermo).
3. dNTPs, 10 mM each and 10 mM total (Thermo),
4. RNase Out (Thermo).

5. Second-strand buffer (Thermo).
6. *E. coli* DNA ligase (NEB).
7. *E. coli* DNA Pol I *E. coli* (NEB).
8. Agencourt AMPure beads.
9. Magnetic stand (for bead cleanup).
10. Bead-binding buffer: 20% PEG, 2.5 M NaCl.
11. T7 In vitro transcription kit (NEB).
12. RNase H (NEB) ExoI (NEB).
13. rSAP (NEB).
14. Fragmentation buffer: 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc.
15. Fragmentation stop buffer: 0.5 M EDTA.
16. Agencourt RNAClean XP beads.
17. Qubit RNA high sensitivity assay kit (Invitrogen).
18. Bioanalyzer RNA pico kit (Agilent).
19. Qubit fluorometer (Invitrogen).
20. Bioanalyzer or TapeStation (Agilent).

2.4 Library Preparation

1. NEBnext High Fidelity 2× PCR Master Mix (NEB).
2. Qubit ds DNA high sensitivity assay kit (Invitrogen).
3. Bioanalyzer DNA high sensitivity kit (Agilent).

3 Methods

The protocol consists of the following steps (*see* also Fig. 1): First, the sample is sectioned into thin slices using a cryostat. Then RNA from individual slices is extracted. Afterwards, a slightly modified version of the CEL-seq2 [8, 9] protocol is used to prepare sequencing libraries with slice-specific DNA barcodes.

3.1 Sample Preparation

1. Embed zebrafish embryo of desired stage in a cryomold filled with tissue-freezing medium. Under a dissection microscope, orient the embryo to the axis that you want to section along (*see* **Note 1**).
2. Snap freeze the tissue block by placing it on top of an aluminum rack on dry ice. The sample is completely frozen when it appears matt white. Label the edge of the cryomold where you want to start sectioning with a pen, and mark the corresponding edge of the block by adding a small notch with a razor blade.

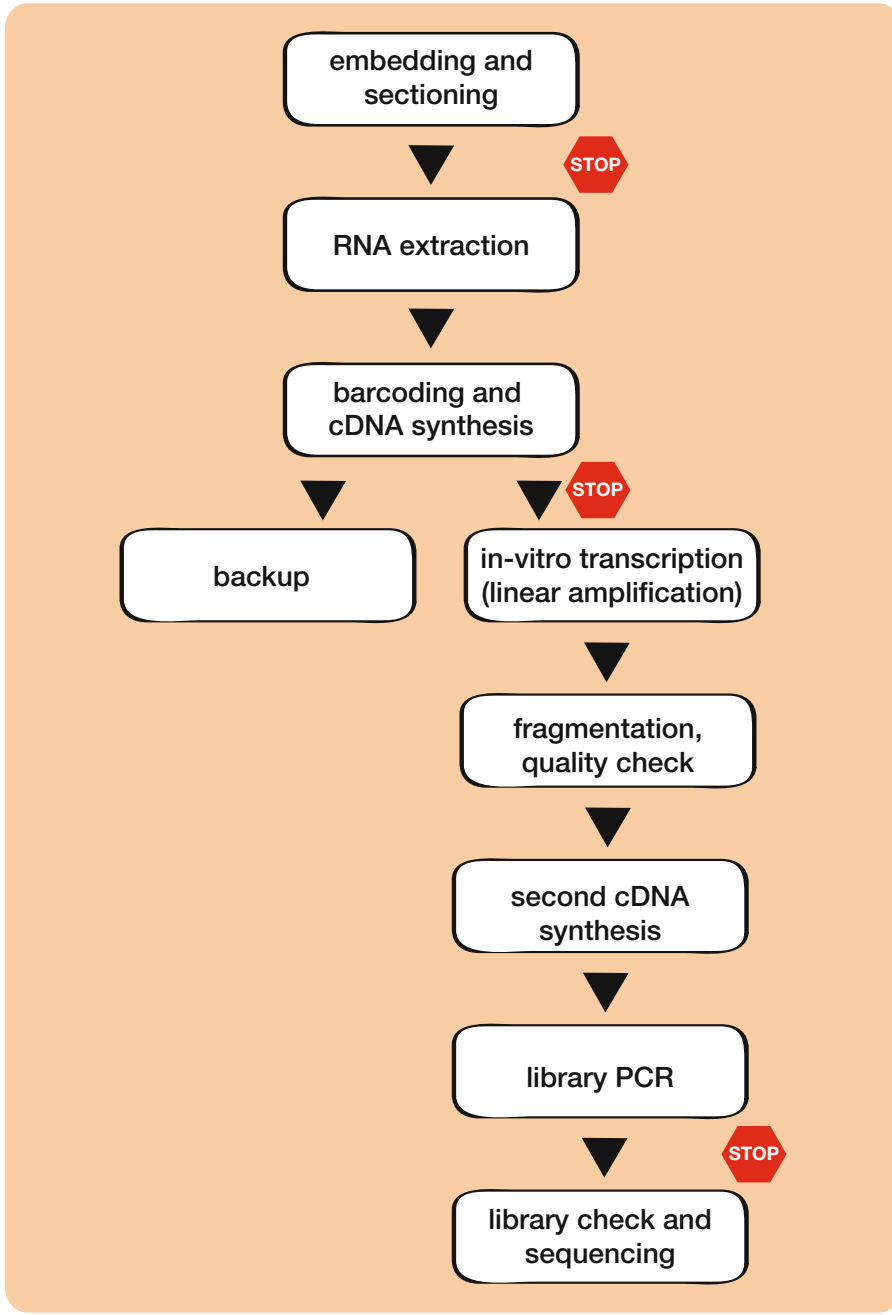

Fig. 1 Flow diagram of individual steps of the tomo-seq protocol. Steps are indicated as boxes connected by arrows. Stopping points are indicated by red STOP signs—samples can be stored at −20 °C for at least several weeks

3. Prepare a sample holder with cryomedium, and place the frozen tissue block in the proper orientation. Snap freeze on dry ice or the cryostat. Prepare a rack with consecutively numbered RNase-free 1.5 mL tubes and cool on dry ice (*see* **Note 2**).

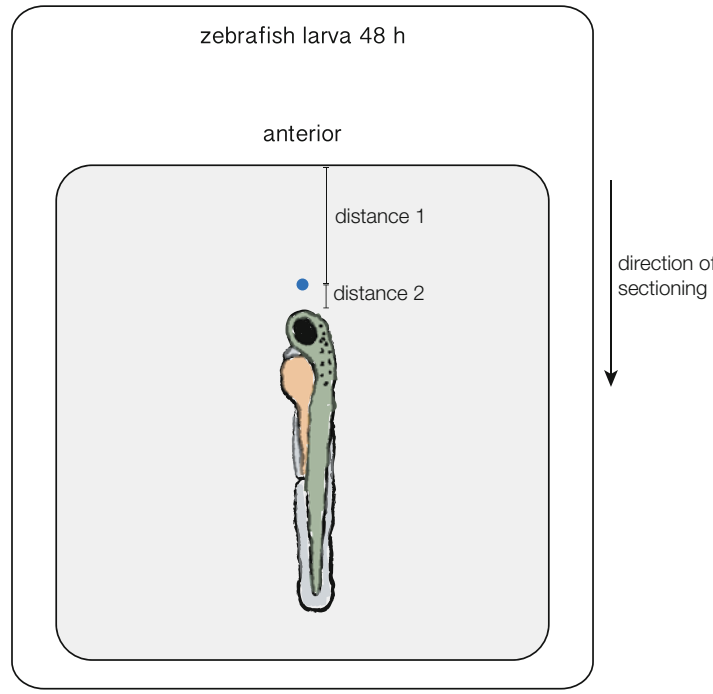

Fig. 2 Sample preparation. Zebrafish larva is transferred into a cryomold filled with tissue-freezing medium and oriented perpendicular to the direction of sectioning. To mark the starting point for collection of individual sections, a small bead is positioned near the embryo with tungsten needles. A picture of the samples is acquired to calculate the indicated distances 1 and 2, respectively. Then the sample is quickly frozen on dry ice and labeled

4. Section the tissue block on a cryostat at desired thickness. We recommend 8–10 μm thickness in a total of up to 96 sections. For larger samples section thickness may have to be increased. During the cutting process, let the individual sections roll up. Transfer them to individual prechilled tubes with a toothpick. Discard empty sections (distances 1 and 2 in Fig. 2; *see* **Note 3**).

3.2 RNA Extraction

1. To each tube, add 0.5 mL TRIzol reagent, 0.5 μL GlycoBlue, and 1 μL of 1:50,000 diluted ERCC spike-in RNA (*see* **Note 4**). If available, use multistep pipette. Use a well-mixed master mix to ensure correct concentrations of spike-in RNA and GlycoBlue (*see* **Note 5**).
2. Close tubes tightly and shake vigorously for 15 s. Incubate at room temperature for 5 min. Samples can be frozen and stored at −80 °C at this point.
3. Add 100 μL chloroform to each tube, shake vigorously for 15 s, and incubate at room temperature for 5 min. Centrifuge at 12,000 × *g* for 15 min at 4 °C (*see* **Note 6**).

4. Remove 250 μL of the colorless aqueous phase by angling the tube at 45° and transfer into a fresh tube. Avoid contaminating the RNA with the interphase consisting of tissue-freezing medium.
5. Add 250 μL isopropanol to each sample, shake vigorously, and incubate overnight at −20 °C.
6. Centrifuge samples at 12,000 × *g* for 10 min at 4 °C. A small blue pellet should be visible in each tube (*see* **Note 7**).
7. Remove the supernatant without disturbing the pellet. Wash with 500 μL freshly prepared 75% ethanol. Samples can be stored at −20 °C in 75% ethanol.

3.3 cDNA Synthesis and First Amplification Step

1. Prepare primer/dNTP mix for each barcoded primer:

Primer (25 ng/μL)	1 μL
dNTPs (10 mM each)	0.5 μL
Nuclease-free H_2O	3.1 μL

2. Remove as much ethanol as possible, and spin down to collect all the liquid at the bottom of the tube. Use a P10 pipette to carefully remove the remaining ethanol.
3. Air-dry pellet on room temperature for a few minutes in aluminum racks until any remaining traces of ethanol have evaporated. Process 4–10 at the same time.
4. When pellets are dry, add 1 μL of barcoded primer/dNTP mix, respectively, and put the samples back on ice.
5. Critical: Incubate RNA/primer mix at 65 °C for 5 min. Meanwhile prepare master mix for the first reverse transcription (first-strand mix): 1 μL per section, 5% reserve (for 96 sections prepare a 100× master mix). After incubation, cool down samples on ice for at least 1 min.

First-strand mix	1 section
First-strand buffer	0.4 μL
DTT 0.1 M	0.2 μL
Nuclease-free H_2O	0.2 μL
RNase Out	0.1 μL
Superscript II	0.1 μL

6. Add 1 μL of the reaction master mix to each sample. Mix well, spin down, and incubate at 42 °C for 1 h in a thermal cycler with the lid set to 50 °C. Heat inactivate the enzyme for 10 min at 70 °C.

7. Cool samples below 16 °C by leaving them on ice for 5 min. Prepare a master mix for the second-strand synthesis, and add 11 μL of the second-strand synthesis mix to each sample (*see* **Note 8**).

Second-strand mix	1 Section
Nuclease-free H_2O	7.72 μL
Second-strand buffer	2.5 μL
dNTPs, 10 mM total	0.25 μL
DNA ligase	0.09 μL
E. coli DNA Pol I	0.35 μL
RNase H	0.09 μL

8. Mix well, spin down briefly, and incubate at 16 °C for 1 h in a thermal cycler with the lid set to 20 °C. Stopping point. Samples can be pooled and frozen at −20 °C.
9. Clean up cDNA with AMPure XP beads:
   - Prewarm AMPure XP beads to room temperature.
   - Pool all samples into one tube. You should have ~13 μL from each section.
   - Vortex AMPure XP Beads until well dispersed, and then add, for each 100 μL of pooled sample, 20 μL beads and 100 μL bead-binding buffer.
   - Incubate at room temperature for 5 min; do not use the aluminum rack (*see* **Note 9**).
   - Place on magnetic stand for at least 5 min, until liquid appears clear.
   - Remove and discard the supernatant.
   - Add 200 μL freshly prepared 80% ethanol.
   - Incubate for at least 30 s, and then remove and discard supernatant without disturbing beads.
   - Repeat ethanol wash once.
   - Air-dry beads for 10 min, or until completely dry (*see* **Note 9**).
   - Resuspend with 13 μL nuclease-free water. Mix thoroughly.
   - Incubate at room temperature for 3 min.
   - Place on magnetic stand for 5 min, until liquid appears clear.
   - Transfer 12.9 μL of supernatant to a new tube.

10. Assemble the following mix for the first amplification step:

IVT MIX	1×
cDNA	6.4 µL
T7 buffer	1.6 µL
T7 enzyme	1.6 µL
ATP	1.6 µL
GTP	1.6 µL
CTP	1.6 µL
UTP	1.6 µL

- Incubate the sample in thermocycler at 37 °C for 16 h with lid at 70 °C, and then cool down to 4 °C. RNA is stable for at least some hours.
- Store the remaining cDNA at −20 °C as a backup.

11. Add 3 µL ExoI and 3 µL rSAP to the sample in the IVT mix and incubate for 15 min at 37 °C. This step removes residual barcoded primers.
12. To the resulting 22 µL of amplified RNA (aRNA), add 5.5 µL fragmentation buffer. Heat to 94 °C for 3–6 min in a thermal cycler. Immediately put on ice and stop reaction with 2.75 µL 0.5 M EDTA. Critical: Fragmentation time depends on RNA concentration. Start with a 3-min fragmentation and repeat after **step 14**, if necessary.
13. Clean up aRNA with 1.8× RNA clean beads:
    - Prewarm RNAClean XP beads to room temperature.
    - Vortex RNAClean XP beads until well dispersed, and add to sample 55 µL beads.
    - Incubate at room temperature for 5 min.
    - Place on magnetic stand for at least 5 min, until liquid appears clear.
    - Remove and discard the supernatant.
    - Add 200 µL freshly prepared 70% EtOH.
    - Incubate for at least 30 s, and then remove and discard supernatant without disturbing beads.
    - Repeat ethanol wash two more times.
    - Air-dry beads for 4 min, or until completely dry (*see* **Note 10**).
    - Resuspend with 7.5 µL nuclease-free water.
    - Incubate at room temperature for 2 min.

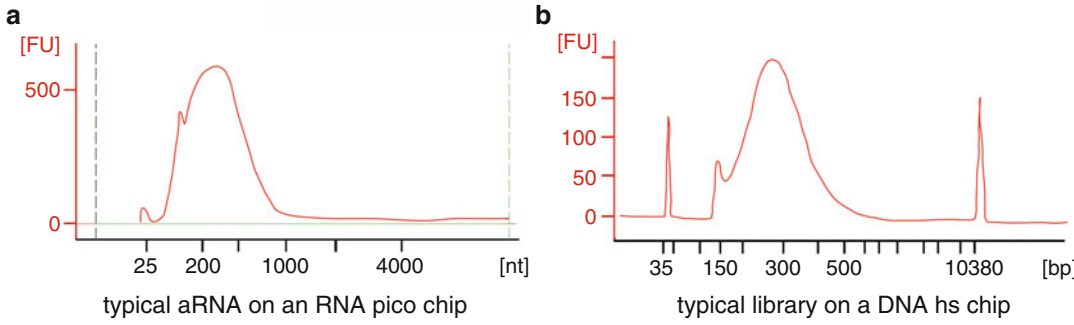

Fig. 3 Output material. After in vitro transcription (IVT), fragmented aRNA is analyzed on a Bioanalyzer RNA pico chip (**a**). Size distribution should peak between 300 and 500 bp, and the fraction of fragments larger than 1000 bp should be minimal. (**b**) The final library size distribution on a Bioanalyzer high-sensitivity chip. The distribution should peak between 300 and 500 bases, and fragments larger than 1000 bp should be negligible. A smaller peak around 150 bp usually derives from primer artifacts and is not problematic for sequencing

- Place on magnetic stand for 1 min, until liquid appears clear.
- Transfer 7 μL of the supernatant to a new tube.

Stopping point. RNA can be kept at −80 °C.

14. Measure RNA concentration with Qubit RNA high sensitivity kit. Dilute 1 μL aRNA to 1 ng/μL and analyze 1 μL of the dilution on a Bioanalyzer RNA pico chip. Size distribution should peak between 300 and 500 bp (Fig. 3) (*see* **Note 11**).

3.4 Library Preparation

1. Dilute aRNA to a maximum of 50 ng/μL in a volume of 5 μL. Add 0.5 μL of the second RT primer (c = 250 ng/μL) and 0.5 μL dNTPs 10 mM total.
2. Incubate for 5 min at 65 °C and immediately put on ice. Cool down for at least 1 min.
3. Add the following reaction mix to the RNA:

Second RT MIX	**1×**
First-strand buffer 5×	2 μL
0.1 M DTT	1 μL
RNase Out	0.5 μL
Superscript II	0.5 μL
Total volume	4 μL

4. Generate cDNA by incubating in a thermal cycler for 10 min at 25 °C, and then for 1 h at 42 °C with lid set to 50 °C, and inactivate for 10 min at 70 °C. Then place the tube on ice.

5. In a final PCR, the Illumina sequencing adaptor and index are introduced. Add the following reagents to the cDNA and mix well by pipetting up and down:

PCR MIX	1×
Nuclease-free H_2O	11 µL
PCR premix NEBnext 2×	25 µL
RP1 primer	2 µL
RPI index primer	2 µL
Total volume	50 µL

Run the following PCR program:

- 30 s at 98 °C
- 12–15 cycles of
  - 10 s at 98 °C
  - 30 s at 60 °C
  - 30 s at 72 °C.
- 10 min at 72 °C, hold at 10 °C.

Stopping point. Samples can be frozen at −20 °C.

6. Clean up PCR product as in **step 9**, Subheading 3.3. Use 45 µL beads and elute in 30 µL.
7. Clean up again as in **step 9**, Subheading 3.3, but use 30 µL AMPure beads and elute in 11 µL nuclease-free water. Stopping point. Sample can be kept at −20 °C.
8. Analyze sample concentration with Qubit DNA high sensitivity assay kit. Concentration should be between 1 and 15 ng/µL (*see* **Note 12**).
9. Examine size distribution of the cDNA library on a Bioanalyzer using a DNA high-sensitivity chip. Library should peak between 200 and 500 bp (Fig. 3). Fragments should not be longer than 1 kb (*see* **Note 13**).
10. Sequence the library paired end on an Illumina platform. The first read (R1) will contain the section barcode and a unique molecular identifier (UMI) [10]. Therefore, the minimum number of cycles for R1 is 16. Read 2 should have a minimum length of 50 bases and will be mapped to the transcriptome later. We typically use a minimum of 500,000 reads per section, so for a library of 96 sections, one would calculate for 48 million reads.

	section 1	section 2	section 3	⋮	⋮	section 95	section 96
ank1	0	1	2	...	...	0	0
ano1	99	2	10	...	...	17	0
apc	0	1	0	...	...	2	0
casp9	3	1	1	...	...	0	6
exd2	0	2	10	...	...	17	0
...	6	8	29	...	...	2	1
...	0	3	10	...	...	30	77
sirt1	2	2	4	...	...	3	2
wnt8a	0	0	1	...	...	10	9
zc4h2	12	27	23	...	...	1	0

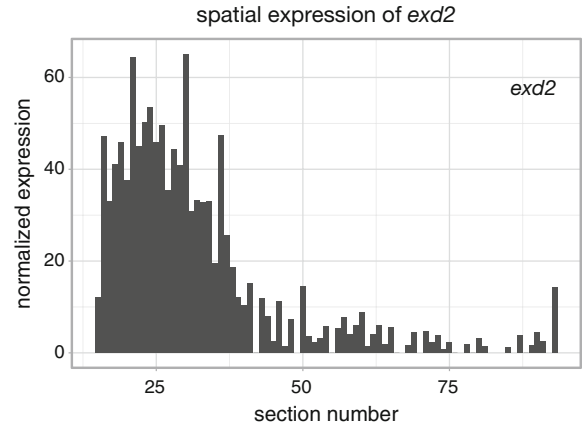

Fig. 4 Data format. The output is a count matrix with rows corresponding to genes and columns corresponding to sections. Columns can be normalized by total counts per section or by ERCC spike-in counts. Spatial gene expression traces correspond to rows in the normalized matrix

3.5 Data Analysis

1. Demultiplex data, and insert the used Illumina indices (*see* RPI sequence information) in the sample sheet.
2. Mapping and counting of the sequencing data are done with custom scripts developed by Grün et al. [11, 12]. Alternatively, the Yanai lab offers their pipeline for CEL-seq2 data analysis at github (https://github.com/yanailab/CEL-Seq-pipeline). In short, R2 is mapped to the organisms' transcriptome, assigned to the barcode of the mate (R1), and then hits per gene and section are counted. The result is a count matrix with rows corresponding to genes and columns to sections (Fig. 4).
3. The data can be normalized by total counts per section or by ERCC spike-in counts in order to ensure correspondence between sections. For analysis in 2D or 3D, expression traces of two or three precisely staged samples sectioned along different axes can be combined computationally [11].
4. Z-score transformed gene expression traces can be clustered to characterize spatial gene expression. Similarity of gene expression can be described by a Euclidean distance ranking [11].

4 Notes

1. For small samples such as early embryos, it may be difficult to correctly identify the first section that contains tissue. To simplify this process, we can use a polyacrylamide bead as a visual marker for the beginning of the sample: Use tungsten needles

to carefully place a single polyacrylamide (PA) bead in the cryomedium between the embryo and the edge that you start the sectioning at, and take a picture under the microscope before freezing. Work quickly to ensure tissue integrity.

2. With a simple image processing program, measure the distance from the edge of the block to the PA bead (distance 1, *see* Fig. 2) in the picture and from the bead to the embryo (distance 2).
3. Make sure that the cryostat and the blade are clean. To prevent cross-contamination with other tomo-seq experiments, move the blade to an unused position. We recommend to set the chamber temperature to −18 °C and the blade temperature to −17 °C. Let the tubes stay in the rack during the transfer of the sections to prevent melting them.
4. During data analysis, spike-in RNA can be used for normalization of different RNA content in single sections. The GlycoBlue reagent coprecipitates with RNA and makes even very small pellets visible.
5. Follow the safety instructions of the TRIzol reagent. Always handle samples in fume hood and wear suitable gloves. Residual TRIzol might be present until **step 11**.
6. Prepare a rack with consecutively numbered 0.5 mL tubes.
7. If samples do not show a blue pellet, add additional 0.5 μL GlycoBlue to these tubes and centrifuge again.
8. At this step, RNA from single sections is already barcoded. Therefore, a single tip can be used for all samples. If desired, up to 8 samples can be pooled for the second-strand synthesis.
9. Use of an aluminum rack can affect the binding of the DNA to the AMPure beads due to a cooling effect.
10. If fine cracks appear, the pellet is overdried. Avoid overdrying, as it affects cDNA yield.
11. If sample shows fragment lengths above 1000 bp, repeat **steps 12–14** of Subheading 3.3. Long fragments will impede library preparation and sequencing of the library.
12. If the library concentration is below 1 ng/μL, repeat the PCR in **step 5**, Subheading 3.4, with three cycles using the same index primer.
13. If the library contains a significant number of fragments longer than 1 kb, check the size distribution of the aRNA and eventually repeat fragmentation. Also check aRNA concentration, and make sure to dilute your samples to a maximum of 50 ng/μL.

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Chapter 10

Optogenetic Control of Subcellular Protein Location and Signaling in Vertebrate Embryos

Clare E. Buckley

Abstract

This chapter describes the use of optogenetic heterodimerization in single cells within whole-vertebrate embryos. This method allows the use of light to reversibly bind together an "anchor" protein and a "bait" protein. Proteins can therefore be directed to specific subcellular compartments, altering biological processes such as cell polarity and signaling. I detail methods for achieving transient expression of fusion proteins encoding the phytochrome heterodimerization system in early zebrafish embryos (Buckley et al., Dev Cell 36(1):117–126, 2016) and describe the imaging parameters used to achieve subcellular light patterning.

Key words Optogenetics, Heterodimerization, Zebrafish, Phytochrome, Polarity, Signaling

1 Introduction

Until recently, the term "optogenetics" has mostly been used to describe the rhodopsin-based control of neuronal firing. However, in the last few years there has been a rapid emergence of many different types of optogenetic systems, mainly based around light-dependent protein dimerization, clustering, or conformational change [1, 2]. These allow researchers to use light to directly control cellular processes such as transcription [3–8], signaling activation [9–16], and full-length protein and organelle localization [17–19]. The choice of optogenetic system will depend on factors such as wavelength compatibility with imaging fluorophores, dynamic range, potential for phototoxicity, and requirements for speed of activation, reversibility, and depth of tissue to be accessed.

I describe here the plant phytochrome heterodimerization system [12], adapted for altering protein localization and signaling within zebrafish embryos in vivo at subcellular resolution [17]. Detailed methods for using this system in mammalian cells are already published [20]. The "anchor" protein phytochrome B

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_10,

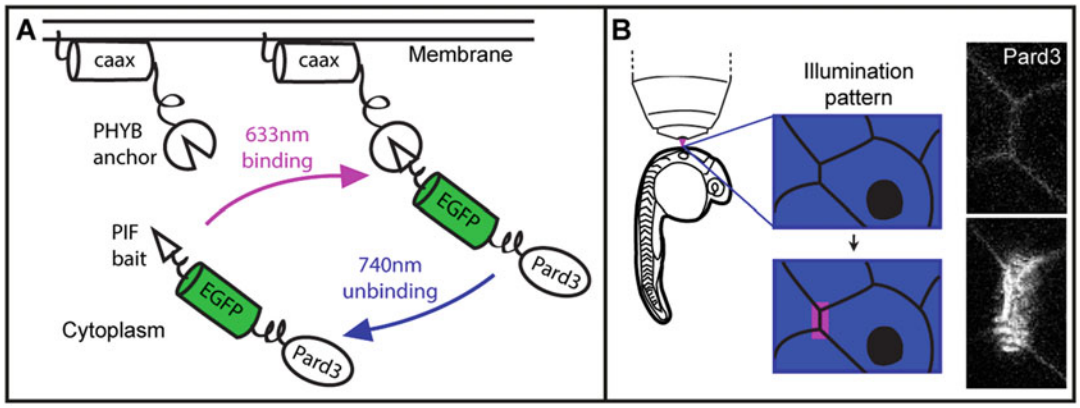

Fig. 1 Phytochrome heterodimerization. (**a**) In this example, the phytochrome B (PHYB) "anchor" protein is linked to the membrane moiety CAAX and the phytochrome interaction factor (PIF) "bait" protein is linked to the EGFP fluorophore and the apical polarity protein Pard3. In the presence of phycocyanobilin chromophore (PCB), PHYB heterodimerizes with PIF under 633 nm illumination, therefore recruiting the fusion protein Pard3-EGFP-PIF6 from the cytoplasm to the membrane. After dimerization, PHYB and PIF proteins remain stably joined unless exposed to 740 nm light, which reverses the dimerization and sends Pard3-EGFP-PIF6 back into the cytoplasm. Both processes occur within a few seconds of light exposure. (**b**) "Binding" 633 nm light is patterned onto a subcellular region of the membrane and is overlaid by global "unbinding" 740 nm light. This results in the precise recruitment of Pard3-EGFP-PIF6 to the region of interest (adapted from Ref. [17] under the terms of the Creative Commons Attribution License (CC BY): http:/creativecommons.org/licenses/by/4.0/)

(PHYB) rapidly heterodimerizes with the "bait" protein phytochrome interacting factor (PIF) under "binding" far-red light (Fig. 1a). Red light penetrates more easily through the tissue and is less damaging than shorter wavelength light. The phytochrome system also has a large dynamic range so only very low levels of light are required to initiate binding. An important property of the system is that dimerization is stable unless actively reversed under "unbinding" near-infrared light (Fig. 1a), which also occurs rapidly (within a few seconds of light exposure). This means that a very high spatiotemporal resolution of optogenetic control can be achieved through the precise patterning of specific "binding" and "unbinding" wavelengths of LED or laser light (Fig. 1b). Therefore, the phytochrome system is particularly suited to experiments requiring the precise optogenetic recruitment of proteins over a long time frame within a 3D tissue, such as in vertebrate embryos. It should be noted that although potentially very powerful, the phytochrome system does require some extra experimental considerations. The high sensitivity of the system means that dimerization will also occur at shorter wavelengths. Any background dimerization can be rapidly reversed with unbinding near-infrared light but careful experimental planning is necessary. Another consideration is that the light-responsiveness of the phytochrome protein is dependent on the photoisomerization of a covalently bound phycocyanobilin (PCB) chromophore. This is not naturally produced by

eukaryotic cells so must be added to vertebrate embryos through microinjection. This adds an additional experimental step but can be advantageous since it makes it easier to produce control embryos containing optogenetic components that are unable to dimerize. A recent paper has now demonstrated the genetic manipulation of mammalian cells to allow endogenous PCB synthesis [21]. This will hopefully be transferrable to living organisms, removing the requirement for external PCB addition in the future. Another group has developed the use of bacterial phytochromes for reversible heterodimerization under near-infrared light [22, 23]. Although the kinetics is slower, this system does not rely on an external chromophore so is promising for future work using red-light-responsive optogenetic systems in vivo.

Although care must be taken with the use of lower wavelength-activated optogenetic systems to avoid phototoxicity, these optogenetic methods are transferrable to vertebrate embryos and most do not require the addition of an external chromophore. Examples of in vivo applications of these techniques are given in Table 1 (this list is not exhaustive). A key difference between these approaches and the phytochrome system is that they mainly do not undergo a stable dimerization change following light illumination and instead revert to their original state in the dark usually over a few minutes (this may or may not be desirable, depending on the output of your experiment). An exception is that cobalamin-binding domain (CBD) systems can produce irreversible optical control of protein complex dissociation, depending on which receptor is being manipulated [29]. Single-cell-level optogenetic manipulation with these systems can be achieved using LED or laser light patterning (e.g., [11]), especially if the targeted tissue is superficially localized. However, if more precise spatial localization is required or the tissue is deeper within the embryo it might be necessary to use two-photon excitation to avoid background dimerization caused by light scattering within the tissue. It is important to note that the biology of the organism can also be used to maximize specificity of optogenetic dimerization. For example, expressing optogenetic components mosaically throughout the embryo will allow only a subset of cells to be activated, even under uniform illumination with binding light. Tissue-specific promoters can further refine the region within the embryo that is sensitive to optogenetic manipulation. Different "anchoring" domains can also be used to increase specificity of dimerization. In the example shown in Fig. 1, PHYB protein is "anchored" to a membrane moiety CAAX. However, "anchor" proteins can be linked to different proteins within the cell, such as motor proteins [18]. This allows the endogenous cell machinery to be hijacked to direct the localization of proteins to particular subcellular regions.

The use of optogenetic dimerization in vivo is evolving and there are many improvements currently under way as well as the

Table 1
Examples of blue and green-light-activated optogenetic systems in vivo

Optogenetic system	Description	In vivo examples
Light oxygen voltage (LOV)	A flavin-binding domain interacts with a Jα helix in the dark and unwinds in blue light. LOV has been variously engineered for allosteric regulation of signaling, heterodimerization, and homodimerization	Photoactivation of Rac1 in zebrafish neutrophils [24]
Cryptochrome (CRY2)	Under blue light, CRY2 protein homo-oligomerizes with itself and also heterodimerizes with CRYPTOCHROME-INTERACTING BASIC HELIX-LOOP-HELIX (CIB)	– Regulation of transcription within the mouse brain using fiber-optic light delivery [4] – Kinase activation in whole Xenopus embryos [25, 26] – Protein kinase C activation in single cells within 4–8-cell-stage early mouse embryos [27]
UVR8	UVR8 homodimers form in the dark and are dissociated into monomers on UV exposure, which can then bind CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1)	Control of ligand secretion in zebrafish leukocytes [28]
EL222	Bacterial LOV protein that binds DNA under blue light	Transcriptional control in zebrafish embryos [5]
TAEL	Modified EL222 system (less toxic)	Transcriptional control in zebrafish embryos [7]
Improved light-inducible dimer (iLID)	Bacterial SsrA peptide within the Jα helix of the LOV2 domain. Under blue light, iLID heterodimerizes with SspB. iLID has been optimized for different binding affinities	Precise control of ERK signaling in early *Drosophila* embryos [11]. This is likely to be adaptable to vertebrate embryos
Cobalamin-binding domain (CBD)	CBD dimerizes in the dark and dissociates into monomers on green-light exposure. Addition of external AdoCbl chromophore is required	Temporal control of overactive FGF signaling in zebrafish [29]

development of new techniques. For example, expressing both components of a heterodimerization system on a single plasmid enables the relative concentrations between dimerizing proteins to be more consistent and has been found to improve the efficiency of optogenetic activation [25]. The generation of stable transgenic animal lines also produces more reproducible levels of protein expression. However, for studies in early embryos, rapid results can be generated by injecting messenger RNA encoding the separate optogenetic proteins into an individual cell during very early development (up to the 64-cell stage in zebrafish, *see* **Note 1**). Injecting at the 8–64-cell stage results in the mosaic distribution of optogenetic components throughout the embryo. Here I present methods for this simple RNA-based optogenetic approach

using the phytochrome system. I describe how to clone plasmids, synthesize RNA, inject RNA into early embryos, mount embryos for imaging, and pattern light using a confocal laser scanning microscope.

2 Materials

2.1 Plasmid Cloning (See Note 2)

1. PSC2+ vector plasmid.
2. Restriction enzymes specific to your plasmid (e.g., ECORI and SnaBI for pCS2+ vector).
3. Electrophoresis reagents: Agarose, TAE buffer, microwave, fume hood, SYBR safe, ladder, loading dye, electrophoresis machine and tank, comb, UV transilluminator, UV protective face shield.
4. Gel extraction kit and razor blades.
5. Primers (*see* **Note 3**): 5′ ends should have 30 bases overlap with the intended upstream sequence. 3′ ends should have a 18–25 base gene-specific sequence.
6. Template for primers (either cDNA or another plasmid containing the DNA of interest).
7. High-fidelity PCR kit.
8. PCR machine.
9. DNA purification kit and DPN1 enzyme.
10. Gibson assembly solution (*see* **Note 4**).
11. Chemically competent *E. coli* (e.g., TOP10 cells).
12. Heat block containing water or water bath at 42 °C.
13. Pre-warmed (37 °C) LB-agar selective plates with ampicillin.
14. LB-broth with ampicillin.
15. Miniprep and midiprep kits.

2.2 mRNA Synthesis

1. pCS2+ plasmid containing DNA template generated in Subheading 2.1.
2. RNase-free water (not DEPC treated).
3. NotI restriction enzyme and buffer.
4. Electrophoresis reagents (as in Subheading 2.1).
5. Sodium acetate (3 Molar).
6. Molecular biology-grade ethanol.
7. Microcentrifuge.
8. Capped RNA transcription kit for the relevant polymerase (SP6 if you are using the pCS2+ vector).
9. RNA cleanup spin column kit.

2.3 mRNA Injection

1. mRNA encoding phytochrome B (PHYB) and phytochrome interacting factor (PIF)-tagged proteins, generated in Subheading 2.2.
2. RNase-free water (not DEPC treated).
3. HPLC-purified PCB chromophore (protect from light): We buy this from SiChem, which is so far the only commercially available source with reliable levels of PCB purity. Dissolve this in anhydrous DMSO (*see* **Note 5**) to a stock concentration of 25 mM and store in 5 μL light-protected aliquots at −80 °C (*see* **Note 6**).
4. Fine loading tips.
5. Glass capillaries (1.0 mm outer diameter, 0.78 mm inner diameter, with filament.)
6. Micropipette puller.
7. Needle-holding plate.
8. Fine-tipped watchmaker's forceps.
9. Injection plate: We use a glass slide inside a large petri dish. Alternatively, you can use an agarose mold to make agarose channels within a large petri dish.
10. Micromanipulator.
11. Microinjector and pump.
12. Graticule and mineral oil.
13. Dissecting microscope.
14. Petri dishes.
15. Aquarium water or embryo medium: Final 1× concentration is NaCl: 5 mM, KCL: 0.17 mM, $CaCl_2{\cdot}2H_2O$: 0.33 mM, $MgCl_2{\cdot}6H_2O$: 0.33 mM, HEPES free base: 5 mM.

    To make up a 60× stock solution, weigh 17.53 g NaCl, 0.76 g KCl, 2.9 g $CaCl_2{\cdot}2H_2O$, 4.9 g $MgCl_2{\cdot}6H_2O$, and 71.49 g HEPES. Add the salts to 900 mL of pure water. Adjust the pH to 7.4 with 10 M NaOH. Top up to 1 L with pure water and check the pH. Filter-sterilize the stock into an autoclaved bottle. Store in the fridge. Once diluted to 1× working solution the pH should be approximately 7.2.
16. Aluminum foil.
17. Heat/cool incubator at 28.5 °C.

2.4 Embryo Mounting

1. MS-222 (tricaine): Make a 25× stock solution of tricaine:

    Tricaine: 0.4% w/v, 1M Trizma base (not with HCL): 2.08%. In the fume hood, weigh 4 g of tricaine powder into a 1 L glass bottle. Add 20.8 mL of 1 M Trizma base (not with HCL). Add 800 mL pure water. Check that PH is 7.0 and adjust if necessary using NaOH or HCL. Make up to 1 L with pure water.

Store in aliquots at −20°C. Use at a 0.016% working concentration (4 mL in 100 mL system water or embryo medium).

2. Fine-tipped watchmaker's forceps.
3. Low-melt agarose: Make up 1% agarose in embryo medium (Subheading 2.3). Store in aliquots in the fridge. To melt, heat to 70 °C in heat block to melt and then cool to 50–55 °C for use.
4. Heat block.
5. Glass pipette and bulb.
6. Imaging dish.
7. Aquarium water.
8. Dissecting microscope.
9. External 740/750 nm light source (*see* **Note 7**).

2.5 Live Imaging and Optogenetic Subcellular Localization of Proteins

1. Confocal laser scanning microscope (LSM) with 633 nm laser line or external light patterning system containing 625 and 740 nm LEDs attached to either a LSM or a spinning disk microscope.
2. Dark imaging chamber with temperature control.
3. External 740/750 nm light source (*see* **Note 7**).
4. Long working distance, high numerical aperture (NA) water immersion objective (*see* **Note 8**).

3 Methods

3.1 Plasmid Cloning (See Note 2)

1. Choose sequences encoding appropriate proteins to link to the PHYB "anchor" and PIF "bait." For example, if you want to activate a GTPase then choose a membrane moiety such as CAAX to link to PHYB (*see* Fig. 1a) and the relevant guanine exchange factor (GEF) to link to PIF (*see* **Note 9**).
2. Design your PHYB- and PIF-containing plasmids using gene visualizing software. Your PHYB-linked protein should either be unlabeled or be labeled with a longer wavelength fluorophore (*see* **Note 10**). The PHYB protein should only be tagged at the 3′ end, never at the 5′ end, which tends to inhibit function of the protein. You should use the shortened version of the PHYB protein (1–621 amino acids of Arabidopsis Gene ID 816394, codon optimized). Your PIF-linked protein should be labeled with a shorter wavelength fluorophore (green or blue-light responsive) to minimize background activation of the phytochrome system. PIF proteins can be tagged at either the 5′ or the 3′ end. Codon-optimized PIF6 (1–100 amino acids of Arabidopsis Gene ID 825382) or PIF3 (1–132 amino acids of Arabidopsis Gene ID 837479) can be used.

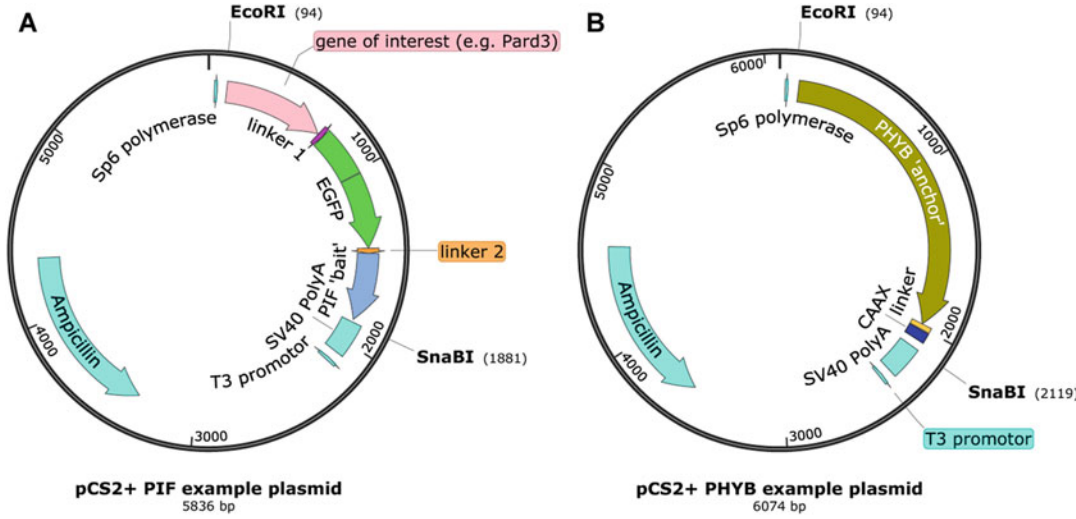

Fig. 2 Example plasmid maps. (**a**) PIF-linked and (**b**) PHYB-linked fusion proteins within the PCS2+ vector

Linkers (such as 10 amino acid polyglycine-serine) should be used between adjacent genes to allow for protein folding. Sequences for these fusion proteins should be incorporated into the pCS2+ vector plasmid between the restriction digest sites ECORI and SnaBI (Fig. 2; *see* **Note 11**).

3. If you are using Gibson assembly (*see* **Note 2**), find templates encoding all the proteins that you want to combine into your plasmid and design and order primers to produce gene fragments with 30 base pair overlapping ends.
4. To prepare the vector (**steps 4** and **5**), linearize the pCS2+ vector at two distinct sites using restriction enzyme digest: Incubate 1–5 μg of pCS2+ plasmid DNA with ECORI enzyme and buffer to a total volume of 20 μL at 37 °C for 2–3 h in a 1.5 μL microcentrifuge tube, as per the manufacturer's instructions. Purify the linearized DNA in the tube using a DNA purification kit, as per the manufacturer's instructions. Further digest this purified DNA as above, using SnaBI enzyme and buffer.
5. Run the whole digest onto a 1% electrophoresis gel: For a 1% gel, add 1 g of agarose to every 100 mL TAE buffer and melt in the microwave. In the fume hood, add 3 μL of SYBR safe and pour into the gel tank containing a comb. Leave until agarose is set. Add ladder to one well and DNA sample with the appropriate amount of gel-loading dye and water into 1–2 other wells. Run the electrophoresis machine as per the manufacturer's instructions at around 100 V for 20 min. Wearing a UV protective face shield and lab coat, visualize the gel on the UV transilluminator and cut out the appropriate weight of

band using a razor blade. Place the gel containing the band into a 1.5 mL microcentrifuge tube and extract the DNA using a gel extraction kit, as per the manufacturer's instructions.

6. Use the high-fidelity PCR kit to amplify your gene fragments using your primers and template DNA, as per the manufacturer's instructions (*see* **Note 12**).
7. Remove residual template from your PCR product either by Dpn1 digestion in the tube or via gel extraction, as above (*see* **Note 13**).
8. Mix together your DNA fragments in a total volume of 5 μL with a molar ratio of 2:1 for insert fragment:vector backbone (*see* **Note 14**). Add this to 15 μL of Gibson assembly solution and incubate at 50 °C for 1 h.
9. Transform the Gibson assembly reaction into chemically competent cells: Thaw 50 μL competent cells on ice. Still on ice, add 5 μL of Gibson assembly reaction to the cells and incubate for 20–30 min. Heat shock the cells at 42 °C without shaking and then immediately transfer back to ice. Spread the cells onto two pre-warmed ampicillin-selective plates and incubate overnight at 37 °C.
10. Place the plate in the fridge in the morning. In the afternoon, pick 4–6 colonies and add each one to 4 mL of LB-broth containing ampicillin within a 14 mL Falcon tube. Shake at 37 °C overnight.
11. Mix 750 μL of each *E. coli* suspension with 750 μL glycerol solution (50% glycerol, 25% nuclease-free water, 25% PBS) and store at −80°C.
12. Extract the plasmid DNA from 1.5 mLs of each *E. coli* suspension using a miniprep kit, as per the manufacturer's instructions.
13. Carry out diagnostic restriction enzyme digests on a sample of each plasmid and run on an electrophoresis gel, as above, to check that you have made the expected plasmid. Send a sample for sequencing to confirm that there are no mutations.
14. Take the glycerol stock from the miniprep with the correct sequence and scrape some frozen cells with a sterile pipette tip. Drop this tip into 2 mL of LB-broth in a 14 mL Falcon tube. Grow at 37 °C in the shaker over the day. Add this 2 mL culture to 150 mL of LB-broth containing ampicillin in a sterile flask and grow at 37 °C in the shaker overnight. Extract the plasmid DNA using a midiprep kit, as per the manufacturer's instructions.

3.2 mRNA Synthesis

1. Linearize the DNA template at a single site using Not1 restriction enzyme digest as in Subheading 3.1, **step 4** (*see* **Note 15**):

2. Check that the linearization has occurred properly: Remove 1 μL of digest and run on an electrophoresis gel (as in Subheading 3.1, **step 5**). Wearing a UV protective face shield and lab coat, visualize the gel on the UV transilluminator to determine whether you have a single band of the expected weight.
3. Ethanol precipitate the DNA (*see* **Note 16**): Add 81 μL of RNase free water to make up to 100 μL volume. Add 10 μL of 3 molar sodium acetate and 400 μL of 100% ethanol. Place at −20 °C for at least 1 h. Using the microcentrifuge, spin at 14,000 × *g* for 10 min. Carefully remove and discard the supernatant, taking care not to remove the pelleted DNA at the bottom of the tube. Add 500 μL of 70% ethanol and spin for a further 5 min at 14,000 × *g* (*see* **Note 17**). Carefully remove and discard the supernatant. Leave the top of the microcentrifuge open and dry the pellet at room temperature. Resuspend the DNA pellet in 6 μL RNase-free water.
4. Synthesize capped mRNA using the RNA transcription kit as per the manufacturer's instructions (*see* **Note 18**).
5. Purify the RNA using an RNA cleanup spin column kit as per the manufacturer's instructions (*see* **Note 19**).
6. Store in 0.5–1 μL aliquots at −80 °C (*see* **Note 20**).

3.3 mRNA Injection

1. Place a glass capillary inside the micropipette puller and adjust settings to pull an injection needle with an approximately 0.5 μm tip. The specific settings will vary by individual machine (*see* **Note 21**) but an example for a Sutter P-87 machine is as follows: heat = 800, pull = 65, velocity = 55, and time = 210. Place the injection needles in a holding plate until ready to load (*see* **Note 22**).
2. Mix the PhyB- and PIF-tagged mRNA and PCB ready for injection (*see* **Note 23**). The exact concentration of RNA will depend on what you are injecting but a rough guide is to inject approximately five times more PHYB-encoding RNA than PIF-encoding RNA (*see* **Note 24**). You should start the working concentration for PCB at 3 mM and adjust it according to how much volume you are injecting and the outcome of your pilot experiments. The injection mix should ideally be used at room temperature right away.
3. Under a dissecting microscope, use forceps to line up around 20 zebrafish embryos in the injection plate and remove excess water.
4. Under low-light conditions load the RNA/PCB mix into the injection needle using a fine loading tip.
5. Insert the needle into the micromanipulator attached to a microinjector and pump.

Fig. 3 RNA injection. Injecting RNA into a single cell within an 8–32-cell-stage embryo results in the mosaic distribution of fusion proteins throughout the embryo. It is possible to target expression to particular regions of the embryo to some extent. We find that targeting the central-most cells results in widespread (but not exclusive) expression in the brain

6. Use fine-tipped forceps to break the last 1–2 mm of the needle taper so that the tip is approximately 6 μm wide.
7. Working quickly to minimize exposure to light (*see* **Note 25**), use a graticule to measure the drop size generated by the microinjector and adjust it to your required drop size (*see* **Note 26**).
8. Use the micromanipulator to insert the needle into a single cell of an 8–32-cell-stage embryo and inject a single drop into the cell (Fig. 3).
9. Place the injected embryos in embryo medium in a petri dish and wrap the dish in aluminum foil to protect it from light. Incubate at 28.5 °C until ready for imaging.

3.4 Embryo Mounting (See Note 27)

1. When the embryos are at the right stage for imaging use fine-tipped forceps to remove their chorions.
2. Using a glass pipette and bulb suck up the embryo in a small amount of water.
3. Drop the embryo into the melted low-melt-point agarose at 50–55 °C (*see* **Note 28**). Remove the remainder of the water in the pipette and suck up the embryo in agarose.
4. Place the embryo in a drop of agarose inside the imaging dish and orient with forceps under a dissecting microscope. Repeat with any other embryos for imaging.
5. Allow the agarose to set for around a minute and cover the embryos with embryo medium. If the embryos will be 16 h postfertilization (h.p.f.) or above during imaging then add tricaine to the embryo medium to prevent embryos from moving.
6. Wrap the imaging dish in aluminum foil to protect from light until imaging.

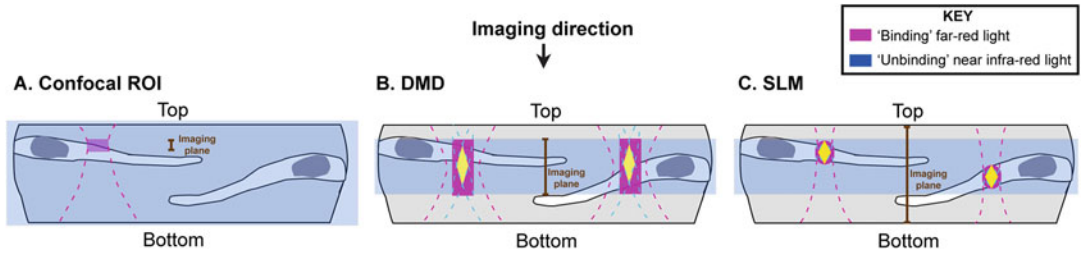

Fig. 4 Patterned light illumination. Transverse diagrams of two zebrafish neuroepithelial cells in the developing neural tube demonstrating three methods of achieving patterned light illumination. (**a**) Using the region of interest (ROI) function of a confocal laser scanning microscope a region of low-intensity 633 nm far-red laser light is patterned onto a single z-plane. This is overlaid with global 740 nm light from an external light source, which prevents any background heterodimerization outside this ROI that might otherwise be caused by the point-spread function pf 633 nm light (dotted lines). This results in a precise region of heterodimerization in xyz but optogenetics and imaging cannot occur outside this single z-level. (**b**) Using a digital mirror device (DMD), a specific ROI is illuminated with 625 nm light at a single z-plane and surrounded by an inverted pattern of 740 nm light. Since the pattern of binding and unbinding light is inverted it is possible to move the recruitment window to a different z-level without reversing binding, therefore allowing small z-stacks to be taken during optogenetics and imaging. However, imaging is limited to the z-planes in which patterned illumination is occurring. Due to the point-spread function of light at each z-level (dotted lines), there will be interference between the two wavelengths, meaning that the recruitment window (yellow diamond) may be smaller than the ROI. (**c**) Using a spatial light modulator (SLM), 633 nm light is directed to specific ROIs over multiple z-planes, while imaging can occur at distinct z-planes. There will be interference between the two wavelengths due to the point-spread function of light at the edges of the ROI, so the recruitment window (yellow diamond) may be smaller than the ROI

3.5 Live Imaging and Optogenetic Subcellular Localization of Proteins (See Note 29 and Figs. 4 and 5)

1. Ensure that the dark imaging chamber on the microscope is preheated to 28.5 °C (*see* **Note 30**).
2. Place your imaging dish onto your microscope while illuminating it with 740/750 nm light.
3. Continuing to bathe the dish in 740/750 nm light, locate the mounted embryos using 488 nm epifluorescence light (*see* **Note 31**).
4. Continuing to bathe the dish in 740/750 nm light and using low-intensity imaging light (488 nm or below, *see* **Note 32**), find the cells that you want to target.
5. Allow any background heterodimerization to be reversed under 740/750 nm light and then take an image (or small imaging stack) of your cells (Fig. 5b-ii). Sequentially image lower wavelengths first, followed by any red wavelengths (*see* **Note 33**).
6. Continuing to bathe the dish in 740/750 nm light, define the region of interest (ROI) in which you want heterodimerization to occur (*see* **Note 34**).
7. Set up your imaging program to take a single image (as in **step 5**), followed by a period of patterned heterodimerization light only

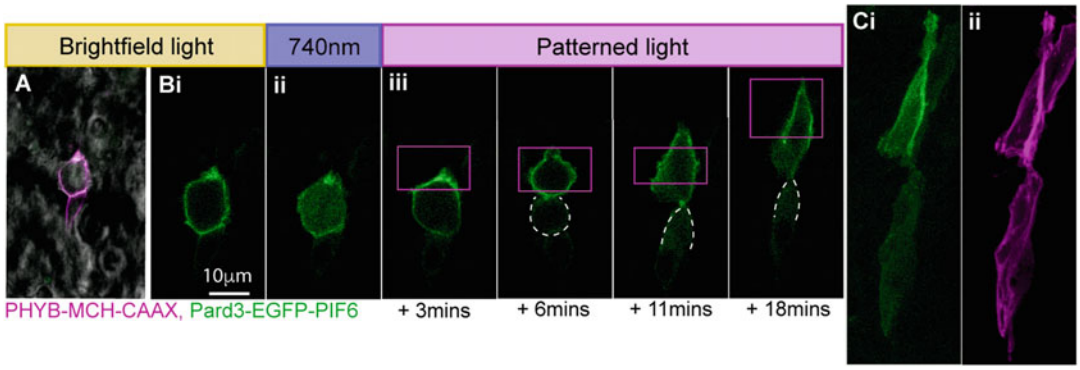

Fig. 5 Example subcellular heterodimerization image sequence. (**a**) Single optogenetically labeled metaphase-stage cell within the zebrafish brain. (**b**) Image sequence of magnified cell from "A" at a single z-level, demonstrating the steps taken to achieve subcellular heterodimerization. (i) Pard3-EGFP-PIF6 was successfully recruited to the cell membrane under bright-field light. (ii) Pard3-EGFP-PIF6 was sent back into the cytoplasm under 740 nm light (90-s exposure). (iii) An ROI is specified (pink box) and illuminated with low-level 633 nm "binding" laser light while 740 nm "unbinding" light is present globally. This patterned light results in the recruitment of Pard3-EGFP-PIF6 to one half of the dividing cell and consequently to the asymmetric inheritance of this protein into the uppermost cell (dotted lines illustrate the location of the lower cell). This is visualized by periodic imaging under 488 nm light. (**c**) After a further 24 min of patterned light illumination at a single z-level, a 23 μm z-stack was taken under 488 and 561 nm light to visualize the distribution of Pard3-EGFP-PIF6 (i) and PHYB-MCherry-CAAX (ii) throughout the depth of the cells (note that there is another pair of cells behind the cells of interest, with low-level Pard3-EGFP-PIF6 expression) (adapted and extended from Ref. [17] under the terms of the Creative Commons Attribution License (CC BY): http://creativecommons.org/licenses/by/4.0/)

(for possible approaches, *see* **Note 29**), followed by another single image. Repeat these steps for as long as necessary for your experiment (Fig. 5b-iii; *see* **Note 35**).

8. Following patterned light illumination you can further image your cells to assess the consequences of the protein recruitment, for example by taking a z-stack (Fig. 5c; *see* **Note 36**).

4 Notes

1. Depending on the protein encoded by the RNA, fusion protein expression in zebrafish usually persists for around 48 h.p.f.
2. You can use whichever method of cloning you prefer. Traditional cutting and pasting using restriction enzymes are possible but not recommended since it is slower than other methods. Some labs use Gateway cloning. We use Gibson assembly [30] in combination with restriction digest of the vector plasmid. (*See* this Jove article for an outline of molecular cloning: https://www.jove.com/science-education/5074/molecular-cloning.)

3. Standard salt purification is fine. Keep to under 60 bases total to reduce costs. Try to adhere to standard primer design guidelines (40–60% GC content, Tm (of the 3′ end only) between 58 and 60 °C, <4°C difference in Tm between forward and reverse primers, no identical runs of nucleotides, no more than two guanines or cytosines at the 3′ end). However, this will not always be possible and usually doesn't matter much.
4. You can buy the Gibson assembly solution commercially or make it yourself [30]. We make our own and freeze it at −20 °C in 15 μL aliquots in PCR tubes, ready for the Gibson assembly reaction.
5. DMSO is highly agroscopic and should be stored in a desiccator to prevent water uptake.
6. PCB seems to be very sensitive to fluctuations in temperature once it is dissolved in DMSO. It is important to ensure that it is stored in part of the freezer where the temperature will be maintained at −80 °C. You can wrap individual aliquots in aluminum foil to ensure that they are protected from light. An alternative would be to use lightproof cryotubes, which may be less sensitive to temperature fluctuations.
7. Near-infrared wavelength light sources are not found on microscopy systems as standard and it is also useful to be able to bathe embryos in "unbinding" light when mounting them before imaging. Therefore, you will probably need to source an external 740/750 nm light source. If you have access to a workshop you can buy 740 nm LED lights and make your own "LED board." Alternatively, you can commission filter glass for use with a white light source. We bought a heat-resistant, 28 mm diameter, 740 nm band-pass filter glass from Envin Scientific Ltd., which we use to filter white light from a Schott KL 1500 light source. You can buy 740 nm LEDs (e.g., from Lightspeed Technologies) for integration into microscope systems [9]. It may be possible to buy these as external units. During imaging, you can insert a 725–750 nm long-pass filter into the bright-field light path, making sure that there are no infrared-blocking filters present [9, 20] or an external light patterning system can be used (*see* **Note 29**).
8. This is important to achieve high-resolution imaging throughout the depth of the embryo and to achieve precise XY region of interest (ROI) patterning.
9. It is possible to move full-length proteins using optogenetics (for example, the apical polarity protein Pard3 [17]). However, especially when manipulating cell signaling, it is better to use the minimal possible component of the pathway in order to activate endogenous pathways with minimal overexpression phenotypes. For example, the catalytic DH domain of the

RhoA-specific GEF (LARG) was used to activate endogenous RhoA signaling when brought to the cell membrane [14].

10. We find that untagged PHYB "anchor" protein works more efficiently.
11. You can use either PCR to amplify all the gene fragments or a combination of restriction enzyme digests and PCR amplifications. This is what we do to avoid PCR amplifications of very long sequences. You can use different enzymes to ECORI and SnaBI as long as they produce an insertion site for your gene fragments after the SP6 polymerase promoter site and before the polyA tail.
12. To avoid introducing errors, do not use more than 25 cycles.
13. If you have used plasmid DNA as your template you can carry out a digest with Dpn1 enzyme as per the manufacturer's instructions, which will destroy *E. coli*-methylated DNA but not the unmethylated PCR product. Otherwise you will need to run your PCR products onto a gel and extract them using the gel extraction kit.
14. Even very low concentrations of DNA often work (with lower efficiency); we have used concentrations as low as 6fmol:3fmol successfully.
15. It is also possible to transcribe RNA directly from PCR templates (*see* the manufacturer's instructions in the RNA synthesis kit). The resulting linearized DNA should contain the SP6 polymerase promoter site followed by the sequence to be transcribed and a polyA tail.
16. The RNA synthesis kit will have specific instructions for terminating and precipitating linearized DNA. Some recommend also using EDTA here. The protocol we have listed is an example of a method that we have used but you can adapt this accordingly.
17. Ensure that the microcentrifuge tube is oriented the same way during both spins so that the pellet stays in the same place.
18. We usually transcribe 1 μg linearized DNA. It is usually recommended to assemble the transcription reaction at room temperature and to add the reaction buffer after the water and ribonucleotides are in the tube. We carry out the transcription reaction for 2 h before terminating the reaction with DNase.
19. Phenol-chloroform extraction and isopropanol precipitation can also be used to purify RNA as per the manufacturer's instructions in the RNA synthesis kit. This will produce purer results but is often not necessary so we use the spin column method for ease. You can run a small volume of RNA on an electrophoresis gel (as for the linearized DNA) to check that you have a clean, non-smudgy RNA band.

20. RNA transcription kits are expensive so it is best to store RNA in small aliquots. It is better not to freeze-thaw RNA so dispose of any remaining RNA in each aliquot after use.
21. You cannot just use the same pulling settings for the different machines, even if they are the same make and model. They all pull slightly differently. You will need to adjust the settings according to the manufacturer's instructions. (*See* this Jove article for details on zebrafish embryo microinjection: https://www.jove.com/video/1115/microinjection-of-zebrafish-embryos-to-analyze-gene-function.)
22. We usually just place a roll of BluTac inside a large petri dish and place the side of the needles into this to make sure that the ends do not get damaged. Needles are best used right after pulling since they will be sharper and less likely to be contaminated.
23. Since PCB is dissolved in DMSO the preparation of the injection mix should be done at room temperature to prevent DMSO from freezing. Also, pipetting small volumes of DMSO is problematic so you should not try to pipette anything less than 0.5 μL of PCB.
24. It is important to ensure that there are a higher number of PHYB "anchor" molecules than PIF "bait" molecules so that all PIF6 molecules can bind during heterodimerization. The balance between PHYB and PIF molecules is critical to ensure efficient heterodimerization so precise RNA injection concentration and drop size are important. We usually inject 0.5 nL of a mixture of 500 ng/μL PHYB-CAAX RNA, 70 ng/μL PIF6-EGFP RNA, and 3mM PCB, diluted in RNase-free water.
25. Until the PCB has covalently bound to the PHYB protein it is unstable under light. Therefore, ideally injections should be done in a darkroom with the microscope bright-field light on a dim setting. You can minimize light exposure by injecting batches of embryos with new injection mix. Do not inject for more than a few minutes each time.
26. A drop size of 0.5 nL is appropriate for injecting one cell in an eight-cell-stage embryo. Smaller drop sizes may be required for older embryos. It is worth setting up the injecting settings to approximately the right settings using water injected into mineral oil so that you can spend less time adjusting these settings for your light-sensitive injection mix.
27. The phytochrome system is very sensitive so heterodimerization will occur if embryos are exposed to white light. This heterodimerization is reversed under 740/750 nm light so an external 740/750 nm light source can be used during mounting to reduce background heterodimerization. It is also

possible to mount embryos using 488 nm epifluorescence if it is particularly important to avoid background binding.

28. It is better to keep the agarose temperature as low as possible to reduce damage to the embryos but note that the lower the temperature, the faster the agarose will set during mounting.
29. If you have access to a confocal laser scanning microscope with a 633 nm laser line it is possible to adapt already existing imaging systems to achieve good subcellular recruitment at a particular Z-plane by using a ROI of low-intensity 633 nm laser light (approximately 1%) overlaid with a global 740/750 nm unbinding light from an external light source (*see* Figs. 1b and 5). This was the method used in Buckley et al., 2016. The global 740/750 nm unbinding light will reverse any potential background heterodimerization caused by the point spread function of the 633 nm laser at other z-planes. However, this method requires that the levels of binding and unbinding light be adjusted so that the binding light outcompetes the unbinding light at the region of interest. Also, recruitment is limited to a single z-plane since moving the recruitment window to a different z-plane would result in the global 740/750 nm unbinding light reversing binding in the original z-plane (*see* Fig. 4a).

    An alternative approach would be to use an inverted light pattern (i.e., only binding far-red light in the region of interest and only unbinding near-infrared light everywhere else). For this you need a separate light patterning system. One option is to use a digital mirror device (DMD), which allows the use of an array of micromirrors to precisely focus light in particular 2D patterns. Inverted light patterning using DMDs has been used very effectively for the phytochrome system in cell culture [9]. This approach is still limited to light patterning at single z-planes, but small z-stacks could be taken to achieve recruitment over a 3D area (*see* Fig. 4b).

    Another option is to use a spatial light modulator (SLM) to alter the phase of coherent light, thereby shaping the excitation in 3D. By using a second objective, the imaging plane can be decoupled from the light patterning plane. Also, several different regions of interest can be simultaneously patterned over different z-planes (*see* Fig. 4c). Precise Z-resolution of patterned light can be achieved through the use of two-photon excitation, such as for the cryptochrome system in Drosophila [10]. However, it is still unclear whether the longer wavelengths used by two-photon microscopes are compatible with the red-light-responsive phytochrome system.
30. It is important to use a dark imaging chamber, to prevent background light from reaching the sample.

31. Since you can reverse any background heterodimerization using the 740/750 nm light it may not be important to your experiment to avoid background activity of the phytochrome system before imaging. In that case, you can just set up the imaging under bright-field illumination, which will be easier. When using an RNA-based optogenetic approach there will be some heterogeneity in expression between cells, meaning that in some cells optimal heterodimerization may not occur (because the balance of PHYB and PIF molecules is not optimal). By allowing background heterodimerization you can screen embryos and cells that respond optimally by selecting those in which your protein of interest has already been recruited (e.g., to the membrane, Fig. 5b-i).
32. The high sensitivity of the phytochrome system means that background heterodimerization will occur, even under 488 nm light. This is minimal if laser levels are kept low and 740/750 nm background light is maintained. Less background activation will occur if lower wavelength fluorophores are used for imaging (such as BFP).
33. This is to ensure that any transient background heterodimerization caused by illumination to longer wavelength light does not confuse the signal from your protein of interest.
34. See introduction where we discuss how the biology of the system can be used to further refine the specificity of optogenetic binding, reducing the need for precise light patterning.
35. Ideally the patterned binding/unbinding light should be constantly present and this should be overlaid by periodic imaging at the imaging wavelengths (Fig. 5b-iii). If heterodimerization is occurring optimally, binding between "anchor" and "bait" molecules should be visible within a few seconds. You will need to adjust the time interval between images according to your own pilot experiments. Imaging every minute is a good starting point. You will also need to adjust the location of the ROI as the cells move and grow.
36. As mentioned, background heterodimerization will occur under imaging light, especially when using red wavelengths. Therefore, the type of further imaging that can be carried out following heterodimerization will depend on the setup of your experiment. You could bathe the embryo in global 740/750 nm "unbinding" light and periodically image the location of your protein of interest. This would allow you to assess the consequences of a short-term heterodimerization later in development. However, if you require the persistent subcellular localization of a protein then you will need to continue to illuminate the cells with patterned light (as in Subheading 3.5, **step 7**) until the end of your experiment.

However, if the spatial localization of heterodimerization is determined by the location of the anchor protein, rather than by your spatial light patterning (as discussed in the introduction) then there is no need for the unbinding 740/750 nm light and you can simply carry out a normal time-lapse image of your cells, ensuring that the embryo is illuminated with red light to maximize heterodimerization.

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Chapter 11

Ex Utero Culture and Imaging of Mouse Embryos

Sonja Nowotschin, Vidur Garg, Anna Piliszek, and Anna-Katerina Hadjantonakis

Abstract

Mouse genetic approaches when combined with live imaging tools are revolutionizing our current understanding of mammalian developmental biology. The availability and improvement of a wide variety of genetically encoded fluorescent proteins have provided indispensable tools to visualize cells and subcellular features in living organisms. It is now possible to generate genetically modified mouse lines expressing several spectrally distinct fluorescent proteins in a tissue-specific or -inducible manner. Such reporter-expressing lines make it possible to image dynamic cellular behaviors in the context of living embryos undergoing normal or aberrant development. As with all viviparous mammals, mouse embryos develop within the uterus, and so live imaging experiments require culture conditions that closely mimic the in vivo environment. Over the past decades, significant advances have been made in developing conditions for culturing both pre- and postimplantation-stage mouse embryos. In this chapter, we discuss routine methods for ex utero culture of preimplantation- and postimplantation-stage mouse embryos. In particular, we describe protocols for collecting mouse embryos of various stages, setting up culture conditions for their ex utero culture and imaging, and using laser scanning confocal microscopy to visualize live processes in mouse embryos expressing fluorescent reporters.

Key words Mouse embryo, Ex utero culture, Time-lapse, Imaging, Reporter, Fluorescent protein, GFP, RFP, Confocal

1 Introduction

Over the past 100 years, mouse genetics has been developed into a powerful system for understanding mammalian biology at the molecular level. The mouse is an excellent model organism to study mammalian biology due to its short gestation period, large litter size, small body size, and resistance to infection.

Unlike many other model organisms such as zebrafish and amphibians, which are readily live-imaged as they undergo normal development [1], mouse embryos being mammals develop within the uterus of their mother, making it necessary to closely mimic conditions in the womb during ex utero embryo culture. In combination with the development of fluorescent labeling techniques

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_11,

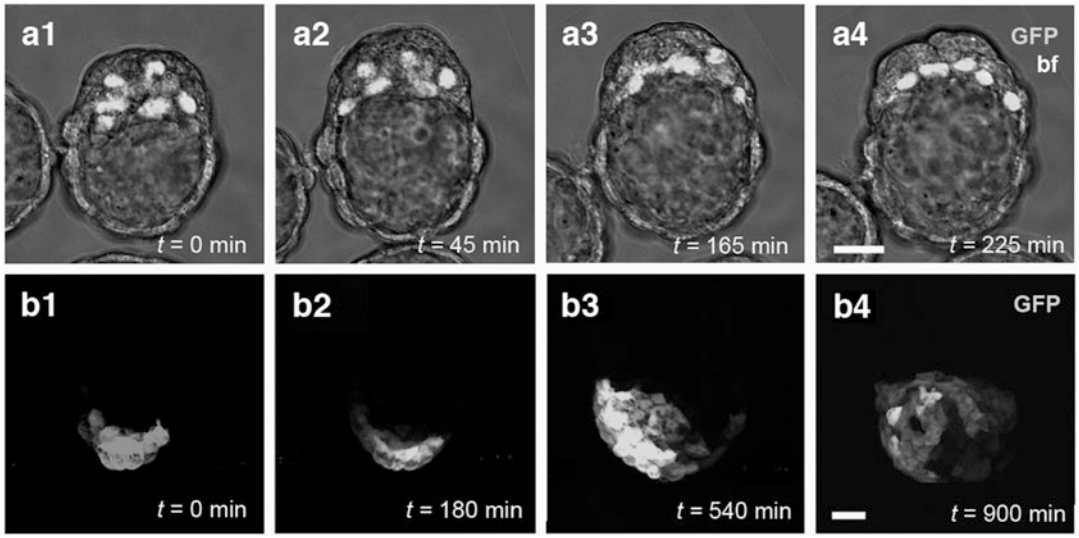

Fig. 1 Examples of 3D time-lapse imaging of mouse embryos. (**a**1–**a**4) Primitive endoderm formation in E3.5-E4.5 embryos; a GFP fluorescent nuclear-localized reporter (H2B-GFP) of Pdgfrα labeling the primitive endoderm population provides single-cell resolution and facilitates cell tracking. (**b**1–**b**4) Anterior visceral endoderm (AVE) migration in E5.5 embryos; cytoplasmic localization of Hex-GFP in AVE cells highlights shapes of migrating cells. All panels represent 3D reconstructions of *z*-stacks taken during fluorescence time lapse. Scale bar: 20 μm

and advances in microscope technology, mouse embryos can now be live-imaged to visualize developmental processes in ex utero-developing embryos.

The characterization and cloning of green fluorescent protein (GFP), originally derived from jellyfish, were awarded the Nobel Prize in chemistry in 2008. Indeed, the discovery and popularization of fluorescent proteins combined with the power of mouse genetics provide attractive tools to follow cells in live organisms [2–6]. Subcellular-localized fluorescent proteins such as the human histone H2B fusion protein (H2B–GFP) labels active chromatin (Fig. 1a), thereby greatly facilitating cell tracking [7–11], while glycosylphosphatidylinositol (GPI) and other membrane-localized fusion proteins help to visualize cell morphology [12–14]. The development of spectrally distinct fluorescent proteins such as the cyan and red fluorescent proteins further facilitates labeling and tracking of different cell populations within the embryo [15–18]. Additional information can be obtained by simultaneously visualizing multiple cellular characteristics, such as cell position (often identified by labeling the nucleus) and cell morphology (by labeling the plasma membrane), which requires the use of multiple subcellularly localized labels. To do this, cells of interest can be dually tagged in various spectral combinations so that for example they express two fluorescent proteins—one localized at the plasma membrane and a second in the nucleus [19, 20].

Lineage-specific expression of fluorescent reporters is an invaluable tool for studying mouse development both in wild-type and experimentally perturbed (for example mutant) embryos and allows for the observation of gene expression in situ in real time (Fig. 1) [21–27]. Single-cell labeling using fluorescent proteins can be achieved either by injection or electroporation of nucleic acids into individual or groups of cells [28, 29] or by using photomodulatable proteins such as KikGR and activating or converting fluorescent proteins in cells in a region of interest [30]. For further information on which fluorescent reporter mouse lines are available within the community, we refer readers to a review by Abe and Fujimori which provides an extensive list [31].

Imaging in bright field with differential interference contrast (DIC) optics has provided useful information of various developmental events, including the timing and plane of cell division in early preimplantation embryos [32, 33], and somitogenesis in later stage embryos [34]. Even though fluorescent probes are highly desirable for multiplex imaging, fluorescence microscopy, while providing a powerful tool for visualizing whole embryos and subcellular structures, introduces the problem of out-of-focus light depending on the thickness of specimens. This problem is partially resolved by image processing and deconvolution techniques. To date, confocal microscopy has been most extensively used for imaging fluorescent probes as it optically sections specimens and eliminates out-of-focus light completely.

Laser scanning confocal microscopy excludes light outside the plane of focus making it possible to optically section a sample, which can be then reconstructed into a 3-dimensional image with the appropriate software. Laser point-scanning confocal microscopes are most commonly used; however other variants are also commercially available. Slit-scanning confocals or Yokogawa-Nipkow-type spinning disk confocals (available from various manufacturers) allow for increased scan speeds and reduced exposure times, and may be preferred for high-speed imaging of rapid processes or for samples that are sensitive to phototoxicity. Multiphoton microscopes also minimize exposure times by illuminating only one focal plane at a time [3, 35]. These advanced optical imaging modalities, combined with optimized ex utero embryo culture protocols and reporter-expressing lines of genetically modified mice, provide powerful tools to live-image dynamic cell behaviors, as well as provide readouts of gene expression, tension, and signaling activity in situ in embryos. Recently, with continually improving embryo mounting chambers and on-stage culture conditions, selective plane illumination (also referred to as light-sheet) microscopy (or SPIM) has become another option to live-image mouse embryos [36–38]. This is augmented by software that can handle the large-file-size light-sheet imaging yields [39]. Furthermore, multi-view light-sheet systems represent a major improvement for

3D microscopy, since the specimen is imaged from multiple angles which are then integrated into a single 3D image, hence overcoming issues of depth penetration for larger samples, and indeed more developmentally advanced embryos [40–42].

2 Materials

All following procedures involving animals must be approved by one's institutional IACUC committee!

2.1 Microscope Setup for Culturing and Imaging Mouse Embryos

1. Stereomicroscope with transmitted light, and both 20× and 40× magnification.
2. Laser-scanning inverted microscope with 5×, 10×, 20×, and 40× objectives (for example PlanApo or PlanNeo objectives). 5× and 10× objectives are usually used dry, 20× are usually used either be dry or multi-immersion, and 40× objectives are usually oil or multi-immersion. 5× Magnification is used for scanning the field of view to identify and position samples. 10× is used for low-magnification 3D time-lapse image acquisition which can give an overview of a developmental process. However, for cell tracking and cell morphology analyses, high-magnification 3D time-lapse imaging is required using at least a 20× objective. Indeed, in most instances a 40× is used to achieve the desired resolution and single-cell magnification. Occasionally a 63× objective may be used for imaging. However, in our experience, this is often times too high a magnification for experiments on even the smallest of mouse embryos (namely preimplantation-stage embryos) or embryo-derived tissue explants.
3. A computer workstation with sufficient RAM and a suitable graphics card with image data acquisition and processing software, such as those available with commercial microscope systems, for example Zeiss's ZEN or Perkin Elmer's Volocity. Additional available platforms for 2D/3D/4D image analysis are the commercially available software packages such as Imaris (Bitplane, http://www.bitplane.com/), or open-source software such as ImageJ or Fiji (NIH, https://imagej.nih.gov/ij/ or https://fiji.sc/). However, such generic software can be challenging to use on semi- or fully automated medium- to high-throughput analyses of multiple samples of sometimes irregular shape and size. For preimplantation embryos, we routinely use the modular interactive nuclear segmentation (MINS) software which we developed in our laboratory specifically for these stages of mouse embryo [43, 44]. MINS performs unsupervised nuclear segmentation of confocal *z*-stacks of multiple samples. It requires minimum optimization of image parameters via a user-friendly interface for nuclear

identification in 3D, providing spatial and fluorescence intensity measurements for all nuclei in large cohorts of embryos at a time [11, 45, 46]. Future versions of this software will likely address issues of the different nuclear shapes and cell nuclei present at pre- and postimplantation stages, which need to be taken into consideration for proper segmentation.

2.2 Culturing and Imaging PreImplantation Mouse Embryos

Culture and manipulation media are commercially available from several companies. These media can also be manually prepared in the laboratory.

2.2.1 Media

1. M2 medium (Millipore).
2. KSOM medium (KSOM + AA, Millipore).

2.2.2 Mice

1. Place 1–2 female mice in a cage with a single male (this is oftentimes referred to as a breeding triangle, or harem breeding). To increase efficiency, females can be inspected for estrus before mating. Embryo donor females should be at least 6 weeks old.
2. Check females the following morning for the presence of a vaginal plug. The day of plug detection is counted as embryonic day 0.5 (E0.5), since mating is assumed to have occurred at the midpoint of the dark period.
3. Dissect out the oviduct or uterus to collect embryos (*see* **Note 1**). Embryos at E0.5-E2.5 are found in the oviduct, while later stage embryos reside in the uterus (*see* Fig. 2 for detailed description).

2.2.3 Embryo Culture

1. Humidified CO_2 incubator.
2. On-stage environmental chamber that provides a stable temperature and gas content required for embryo culture (Fig. 3).
3. Gas mixtures ($CO_2/O_2/N_2$; consult methods section for appropriate selection).
4. Dumont #5 forceps (Inox or Dumostar) (Roboz or Fine Science Tools) and small surgical scissors (Roboz) (*see* **Note 2**).
5. 35 and 60 mm plastic Petri dishes (Falcon).
6. Organ culture dishes—optional (Falcon).
7. 35 mm Glass-bottom dishes (MatTek) or Lab-Tek™ II Chambered Coverglass (Nunc™ by Thermo Fisher).
8. Mouth pipette: Assemble from mouthpiece (HPI Hospital Products Med. Tech., 200 μL tip can be used instead), latex tubing (latex 1/8″ ID, 1/32″ wall, Fisherbrand), and finely drawn glass Pasteur pipette (Fisherbrand), using 1000 μL tip as a connector. Pasteur pipette can be hand-pulled over the flame

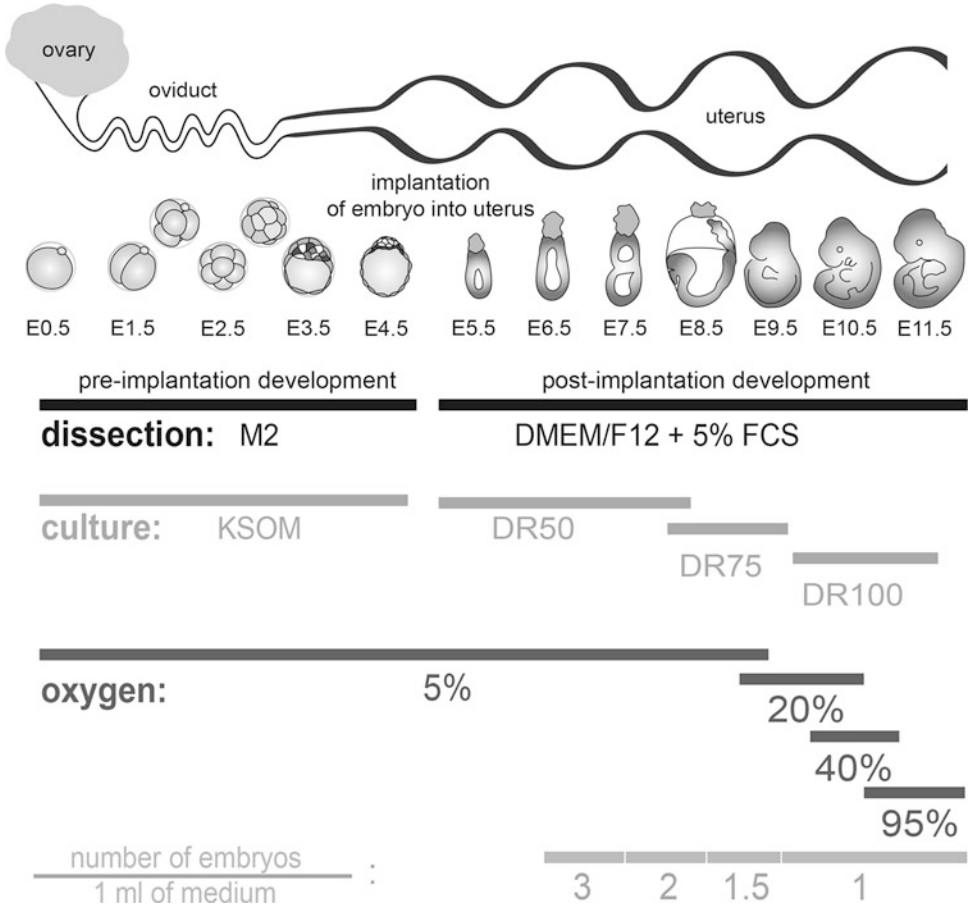

Fig. 2 Schematic representation of time course of mouse embryonic development. Includes requirements for dissection medium, culture medium, and gas content for in vitro culture at each embryonic stage, and approximate location of embryos in reproductive tract at each stage of development. DR = [DMEM/F12 + Glutamax]: rat serum (*see* Subheading 2). Number of embryos/mL for roller culture. Embryo schematics are not to scale

to a diameter of 1.5× the embryo. Pipettes can be siliconized prior to use to prevent embryos from sticking to the glass.

9. 2% Agar (Fisherbrand) + 0.9% NaCl: The glass bottom of the MatTek dish is covered with a thin layer of agar to prevent preimplantation embryos without zona pellucida from adhering to the glass (*see* **Note 3**).
10. Glass rods for preimplantation embryo culture setup (optional, *see* Fig. 4): After pulling a Pasteur pipette over a flame, use the thin filamentous portion of the glass to place on the MatTek dish. Forceps can be used to cut the pulled glass into pieces of ~0.5–1 cm in length to fit the glass portion of the dish.

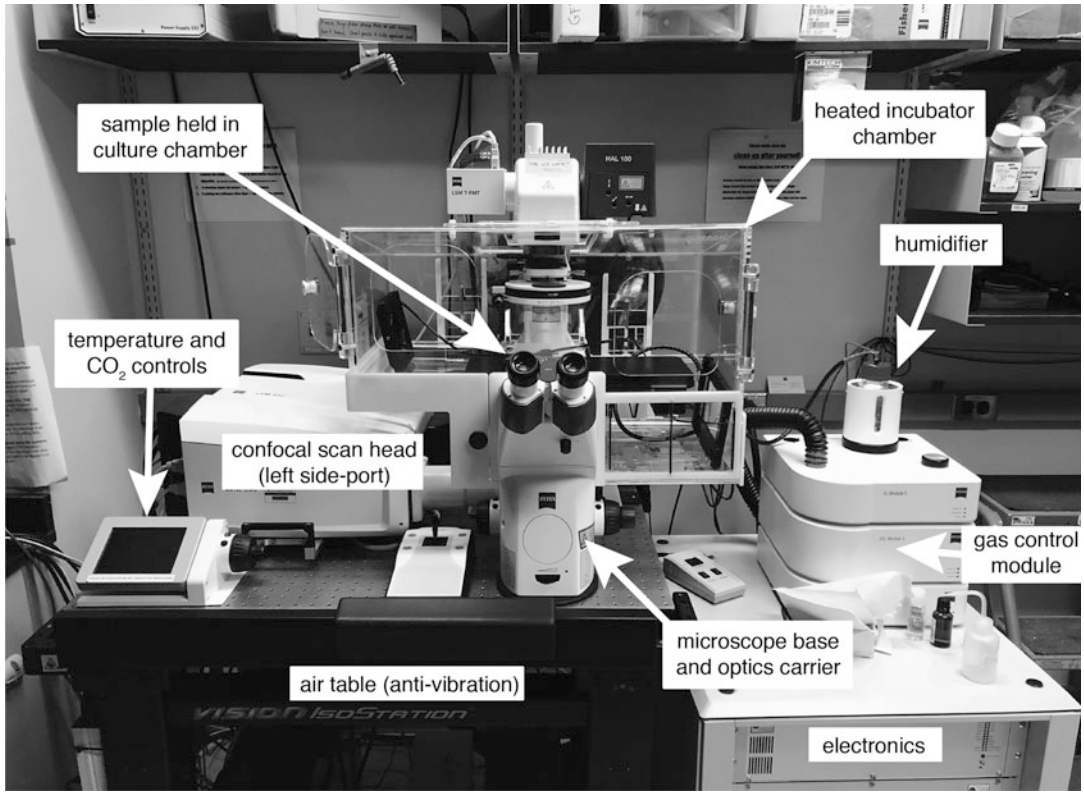

Fig. 3 Microscope setup for live time-lapse imaging. Inverted microscope (photograph shows a Zeiss LSM880 laser-scanning confocal system) fitted with environmental chamber (Zeiss XL1) provides optimal conditions for ex utero culture and live imaging of mouse embryos

11. A 1 mL syringe, 26- or 27.5-gauge needle (Becton Dickinson), and a blunt 30-gauge needle (cut or blunted with sandpaper or sharpening stone) (Becton Dickinson).
12. Embryo-tested lightweight mineral oil (Sigma-Aldrich).

2.3 Culturing and Imaging PostImplantation Mouse Embryos

2.3.1 Media

Culture and manipulation media are commercially available from several companies. These media can also be manually prepared in the laboratory.

1. 95% D-MEM/F12 (1:1) medium (Gibco by Life Technologies) + 5% newborn calf serum (e.g., Gibco by Life Technologies).
2. DR100, DR75, or DR50 (*see* Fig. 2 for specific requirements according to the stage of development): Each medium preparation consists of rat serum, diluted in D-MEM/F12 (1:1) with GlutaMAX (Gibco by Life Technologies). DR100 is pure rat serum, whereas DR75 is 75% rat serum 25% D-MEM/F12, and DR50 is 50% rat serum 50% D-MEM/F12.

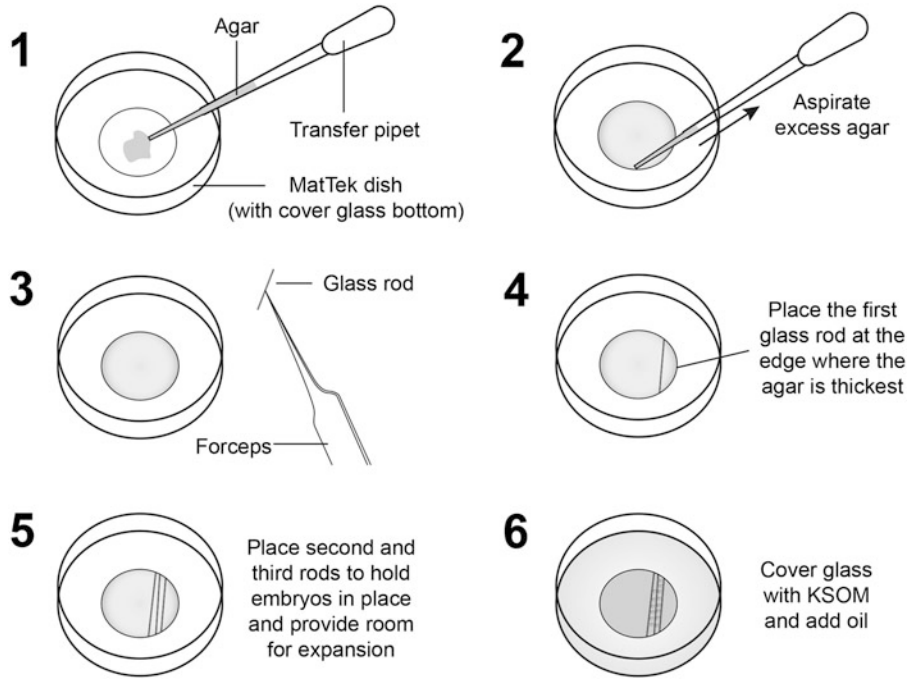

Fig. 4 Immobilizing preimplantation embryos for live imaging. Preparing a culture dish for live imaging of preimplantation embryos using glass rods to secure embryos within the imaging field during culture. (1) Coat the glass bottom of a MatTek dish with agar, and aspirate any excess (2). (3) Carefully place (using watchmaker's forceps) the first glass rod on the edge of the glass using the accumulated agar to hold it in place (4). (5) Place subsequent rods parallel to the first rod, leaving enough space for embryos to fit and expand as they develop. (6) Cover the glass bottom on the dish with KSOM, and subsequently cover the medium with embryo-tested mineral oil (to prevent evaporation)

3. Rat serum (although commercially available, the best results in terms of faithful ex utero embryo development are usually achieved using homemade serum [47]).
   (a) Anesthetize rats (preferably large—retired breeder—males) with Ether or other volatile gas. One can also use isoflurane anesthesia.
   (b) Make an incision in the abdomen and expose the dorsal aorta.
   (c) Gently collect blood from the aorta (12–15 mL per rat), using a syringe.
   (d) Place the tube with the collected blood on ice.
   (e) Euthanize the rat.
   (f) Centrifuge the blood for 20 min at 1300 × *g*.
   (g) Collect the supernatant and remove the pellet.
   (h) Centrifuge the serum for 10 min at 1300 × *g*.
   (i) Collect the supernatant.

(j) Heat-inactivate the serum for 30 min at 56 °C.

(k) Filter the serum with a 0.45 μm filter.

(l) Aliquot the serum and freeze at −80 °C for up to 1 year.

2.3.2 Mice

1. Place 1–2 female mice in a cage with a single male. Embryo donor females should be at least 6 weeks old.
2. Check females the following morning for the presence of a vaginal plug. The day of plug detection is counted as embryonic day 0.5 (E0.5).
3. Dissect out the oviduct or uterus to collect embryos.
4. Stage early postimplantation embryos according to morphological landmarks [48, 49]. The time of dissection should not be used as the criterion for staging of embryos, as a range of stages occur even within a single litter of any given age.

2.3.3 Embryo Culture

1. Humidified CO_2 incubator.
2. Roller apparatus (rotating ~30 rpm) in an incubator chamber (37 °C): A roller culture apparatus providing a constant gas supply is recommended (BTC Engineering, Cambridge, UK).
3. On-stage environmental chamber that provides a stable temperature and gas content required for embryo culture (Fig. 3).
4. Gas mixtures ($CO_2/O_2/N_2$; consult methods section for appropriate selection).
5. Dumont #5 forceps and small surgical scissors.
6. 35 and 60 mm plastic Petri dishes.
7. Organ culture dishes—optional.
8. 35 mm Glass-bottom dishes (MatTek) or Lab-Tek™ II Chambered Coverglass (Nunc™ by Thermo Fisher).
9. Mouth pipette: Assemble from mouthpiece (HPI Hospital Products Med. Tech., 200 μL tip can be used instead), latex tubing (latex 1/8″ ID, 1/32″ wall, Fisherbrand), and finely drawn glass Pasteur pipette (Fisherbrand), using 1000 μL tip as a connector. Pasteur pipette can be hand-pulled over the flame to a diameter of 1.5× the embryo. Pipettes can be siliconized prior to use to prevent embryos from sticking to the glass.
10. Plastic transfer pipettes for moving older embryos (the tips can be cut to increase diameter and accommodate larger embryos; Fisherbrand).
11. Embryo-tested lightweight mineral oil (Sigma-Aldrich).
12. CoverWell™ perfusion chamber (Thermo Fisher or alternatively Grace Bio-Labs by Sigma-Aldrich).
13. Human eyelashes, sterilized with 70% ethanol.
14. Suction holding pipette (optional; Eppendorf CellTram Air).

3 Methods

3.1 Microscope Setup for Culturing and Imaging Mouse Embryos

For live imaging, it is important to combine conditions that allow for embryonic development closely resembling those in utero with a setup that ensures the best image quality. This can be accomplished using an inverted microscope with an environmental chamber setup (Fig. 3) that provides the stable temperature and gas content required for embryo culture [44].

1. With the exception of certain low-magnification bright-field microscopy applications, most objectives are designed to be used with glass coverslips of 0.17 mm thickness (usually referred to as No. 1.5 coverslips). Thus, No.1.5 coverslip thickness MatTek dishes or Lab-Tek chambers should be selected for the types of experiments described in this chapter.
2. Since ultraviolet light and laser beams are particularly harmful to mammalian embryos, imaging conditions should be adjusted for each experiment type (depending on reporters being used, time period for the experiment) to ensure proper development of the embryo. Reducing laser power and exposure time by decreasing the frequency of scans, increasing the size of optical sections, or increasing scan speed can aid the normal development of embryos while still allowing for the good-quality images.

3.2 Culturing and Imaging PreImplantation Mouse Embryos

At the earliest stages of development, mouse embryos float freely along the mother's reproductive tract. Therefore, in vitro culture of preimplantation embryos requires the appropriate medium, temperature, and gas conditions closely resembling those in the womb. These culture conditions are now largely established, allowing for the proper timing and development of preimplantation-stage embryos. For a visual guide for preimplantation embryo culture the authors recommend the video by Saiz et al. [44].

3.2.1 Collection of PreImplantation Mouse Embryos

1. Before starting the dissection of embryos, equilibrate and pre-warm the KSOM culture medium by placing the dish covered with mineral oil for at least 30 min in a humidified incubator at 37 °C (degrees Celsius) and 5% CO_2 in air. Additionally, pre-warm M2 medium at 37 °C.
2. After sacrificing a pregnant female, either (for E0.5-E2.5 embryos) dissect out the oviduct leaving a small part of the distal uterus attached or (for E3.5-E4.5 embryos) remove the entire uterus and place in a drop of pre-warmed M2 medium.
3. Place the dish under a stereomicroscope and flush the oviduct/uterus with pre-warmed M2 medium. Use a 1 mL syringe with a 26-gauge needle (uterus), or a blunt 29- or 30-gauge needle (for the oviduct: insert the blunt needle in the oviduct infundibulum).

4. Collect the embryos and transfer into a culture dish using a mouth pipette attached to a pulled Pasteur pipette (pulled over a Bunsen flame) or capillary pipette (pulled on a horizontal or vertical pipette puller).

3.2.2 Culturing PreImplantation Mouse Embryos

1. Prepare the culture dish by placing drops (10–100 μL each) of KSOM culture medium in the bottom of a 35 mm plastic dish and cover with embryo-tested light mineral oil.
2. Place the dish for at least 30 min in a humidified incubator at 37 °C and 5% CO_2 in air, to allow for equilibration.
3. Transfer the embryos into the microdrops under oil. Transfer through several drops, to rinse off residual M2 medium. Ideally, culture several embryos together, as a higher density of embryos enhances development.
4. Embryos in KSOM medium should only be removed from the incubator for minimal periods of time as the bicarbonate-buffered medium quickly changes pH in the air.
5. Under these conditions, embryos can develop from a zygote to the late blastocyst. If the dissected embryos are at an earlier stage than expected, they can be cultured in vitro until they reach the proper stage without compromising their development, and imaged afterwards.

3.2.3 Live Imaging of PreImplantation Mouse Embryos

Preimplantation embryos are live-imaged under the same conditions as those for static culture. If the levels of CO_2 cannot be reliably maintained, embryos can be imaged short-term at 37 °C in M2 medium instead.

1. Pre-warm the on-stage incubator to 37 °C before live imaging; this can take from 30 min to several hours, depending on the incubator (*see* **Note 3**).
2. Prepare the culture dish by placing a drop of KSOM culture medium in a glass-bottom dish and covering it with embryo-tested light mineral oil (also *see* **Note 4** for additional considerations).
3. Place the dish for at least 30 min in humidified incubator at 37 °C and 5% CO_2 in atmosphere to allow for equilibration.
4. Transfer the embryos into the equilibrated dish. If possible, culture several embryos together even if only one of them is to be imaged. Place the dish on the microscope stage, and immediately provide CO_2.
5. Image the embryos. Minimize embryo exposure to laser light by reducing laser power and exposure time, decreasing the frequency of scans, and/or increasing the size of optical sections and scan speed. These adjustments should be determined empirically and will depend on the individual microscope, and

brightness of the fluorophore. In many cases, 2 μm thick optical sections (up to total of ~100 μm) taken at 7–15-min intervals and combined with low laser power give good results.

6. To prevent embryos from drifting out of the imaging plane, make sure that the microscope stage is leveled. The amount of medium (too much or too little, especially flat drops) may also affect embryo drifting. Placing several embryos together can help to keep them immobile.
7. Additionally, glass rods can be used to prevent embryos from drifting away from the imaging field (Fig. 4). For this, cover the bottom of a MatTek dish with agar and aspirate excess agar so that a thin film remains covering the glass. Excess agar remaining on the edges can be useful to fix the glass rods. Place 2–3 glass rods on top of the agar—closely spaced to hold the embryos in place during imaging. Place the first one with its ends on the edge of the glass where the agar is thicker. Position the second and third rods so that the distance between rods approximates 1.5–2× the width of embryos (provides room for expansion during imaging and development). After setting the rods and placing the embryos between them, cover the glass portion of the MatTek dish with KSOM medium and subsequently cover the medium with embryo-tested mineral oil to prevent desiccation.

3.3 Culturing and Imaging PostImplantation Mouse Embryos

Around E4.0, mouse embryos start to implant in the uterus and begin to form and expand extraembryonic structures, which provide a physical connection with the mother and help support later development. This makes dissection more difficult and creates a unique challenge for ex utero culture once the mother-embryo connection is irreversibly lost. The methods in this chapter describe protocols for embryo culture up until E9.5 (*see* **Notes 5**–**7**).

3.3.1 Collection of PostImplantation Mouse Embryos

1. Before starting the dissection, equilibrate and pre-warm the culture medium by placing the culture dish covered with mineral oil in a humidified incubator at 37 °C and 5% CO_2 in atmosphere for at least 1 h.
2. After sacrificing the female, dissect out the uterus and place in a dish of pre-warmed (25–30 °C) dissecting medium (DMEM/F12 + 5% NCS; Fig. 2).
3. Place the dish under a stereomicroscope, dissect deciduae out of the uterus, and carefully remove embryos from each decidua using watchmaker's forceps. For detailed dissection instructions consult [50].
4. Remove Reichert's membrane from each embryo using watchmaker's forceps. Great care should be taken to avoid damaging embryos in the process of dissection and to ensure that the

ectoplacental cone is left intact. Embryos that have been damaged during dissection should not be used for further culture.

5. Immediately after dissection, carefully move the embryos into a dish of culture medium with a pipette so that only the smallest amount of dissecting medium is transferred.

3.3.2 Roller Culture of PostImplantation Mouse Embryos

Roller culture provides the most optimal ex utero conditions for embryonic development at early postimplantation stages. Using this method, embryos are cultured in controlled temperature and gas conditions and are kept in constant motion.

1. Pre-warm roller culture incubator to 37 °C before onset of culture.
2. Mix culture medium appropriate for the stage of the embryo (Fig. 2). The amount of medium required depends on the stage of the embryo.
3. Equilibrate medium with the gas mixture appropriate for the stage of the embryo (Fig. 2) at 37 °C for at least 1 h before culture. For a roller culture apparatus that has a constant gas supply, place a small amount of medium in the culture bottle within the machine. If this apparatus is not available, blow gas on the surface of the medium using a Pasteur pipette and place in an open dish at 37 °C.
4. Move embryos into roller culture bottles with a pipette. Make sure that only the smallest amount of dissecting medium is transferred. If necessary, wash embryos in culture medium before moving them into culture bottles.
5. Re-gas the tubes, close tightly, and place in the roller apparatus at 37 °C.
6. Re-gas the tubes every 12 h (unless constant gas flow is being provided).
7. Replace the medium with a newly equilibrated mixture after 24 h.

3.3.3 Static Culture and Imaging of PostImplantation Mouse Embryos

Although roller culture provides the best conditions for the ex utero development of postimplantation mouse embryos, it is not suitable for time-lapse imaging. For live imaging, embryos are cultured statically, which normally allows development to proceed for up to 24 h [51]. Atmospheric and temperature conditions for static culture are the same as for embryos subjected to roller culture (Fig. 2).

1. Pre-warm the on-stage incubator to 37 °C before live imaging. This can take from 30 min to several hours, depending on the incubator.

2. Prepare culture medium of composition appropriate for the stage of the embryo.
3. Prepare the glass-bottomed culture dish for imaging. Early postimplantation embryos (E5.5-E8.5) are cultured in drops of medium covered with embryo-tested light mineral oil (*see* **Note 8**). Place the culture dish in a humidified incubator at 37 °C and 5% CO_2 in atmosphere for at least 1 h to pre-warm and equilibrate.
4. Move embryos into the culture dish with a pipette. Make sure that only the smallest amount of dissecting medium is transferred. If necessary, wash embryos in culture medium before moving them to the culture dish (*see* **Note 6**).
5. After moving the dish containing the embryos to the microscope stage, immediately provide CO_2 (*see* **Note 7**).
6. Image the embryos. Minimize embryo exposure to laser light as much as possible by reducing laser power and exposure time, decreasing the frequency of scans, and/or increasing the size of optical sections and scan speed. These adjustments will depend on the microscope and brightness of the fluorophore used as well as the developmental stage of the embryo. Using a standard laser-scanning confocal microscope system (for example a Leica SP8, Zeiss LSM880, or equivalent from a different commercial vendor), bright fluorophores (these are usually empirically tested), 5–7-min time intervals between *z*-stacks, and approximately 2 μm between individual planes within a *z*-stack, should be sufficient to identify (i.e., segment) and track cells from one time frame to another. Since embryonic development is easily perturbed by culture conditions or phototoxicity, careful optimization of experimental conditions on wild-type or heterozygous reporter-expressing embryos that do not have a defect is necessary, before proceeding to analyze mutants exhibiting phenotypes.

3.3.4 Immobilizing PostImplantation Embryos

For some experiments [52] postimplantation embryos may need to be imaged on their distal or ventral side. This can be done by using a variety of methods, including embedding them in low-percentage low-melt agarose or collagen, and suspending them in culture using either a suction holding pipette or modified chamber such as a CoverWell™ perfusion chamber (Fig. 5) [52]. The gaskets of the chambers are cut into fragments containing a silicon body attached to a plastic surface with a hole (gaskets of different thicknesses can be used according to embryo size, and the plastic plate can be bent to position the embryo at different angles, or to accommodate smaller embryos).

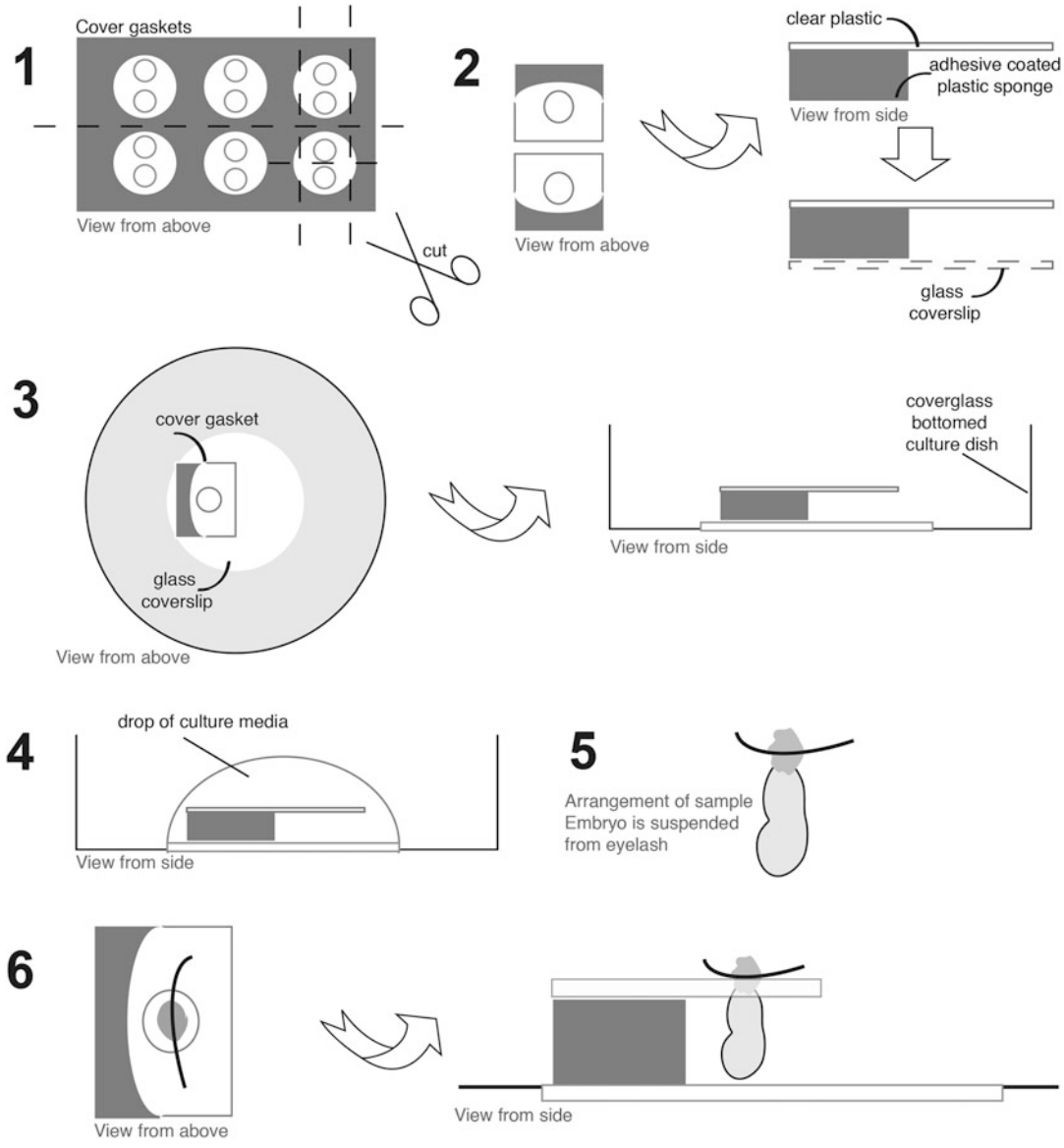

Fig. 5 Immobilizing postimplantation embryos using adhesive perfusion chambers. (1) CoverWell™ perfusion chambers (Molecular Probes) are cut into small pieces, each containing a silicon body attached to a plastic surface with a hole. (2) Bottom plastic cover is removed. (3) A such prepared chamber is placed on a glass-bottom dish with the adhesive side of the silicone part facing down and placed on a hot surface for about 20 min to promote adhesion. (4) Culture medium is added to the dish covering the glass surface and the chamber. Mineral oil is added to the dish to avoid evaporation during culture. (5) The ectoplacental cone of the embryo is pierced with an eyelash. (6) The embryo is suspended through the hole of the chamber

1. Adhere a precut piece of the chamber to a glass-bottom dish and place the dish on a hot surface to facilitate adhesion by melting of the plastic (Fig. 5 (1–3)).
2. Add culture medium to the glass part of the dish (Fig. 5 (4)).
3. Cover the culture medium with light mineral oil.
4. Pierce the ectoplacental cone of the embryo with an eyelash and suspend the embryo through the hole of the gasket. The embryo should hang vertically from the plastic plate (Fig. 5 (5, 6)).
5. Move the embryos to the microscope-stage incubator set at 37 °C and supplied with the appropriate CO_2 and O_2 concentration.
6. Image the embryos.

4 Notes

1. It is imperative to work quickly and efficiently. Prolonged time on the bench adversely affects embryos and compromises their subsequent culture. Therefore, a balance needs to be struck between speed and care. If you have more than one litter to dissect, sacrifice females one at a time.
2. It is recommended to use a set of coarse tools for the dissection of the uterus from the mouse, then one set of less pristine watchmaker's forceps (#5 s) to remove the decidua from the uterus, and a second set of pristine watchmaker's forceps (#5 s) for the dissection of embryos from the decidua. Removal of Reichert's membrane requires particularly fine forceps, which can be sharpened whenever necessary with a sharpening stone.
3. It is necessary to replace water in the incubator humidifier bottle periodically, as the water can become contaminated and affect embryo development. The bottle should be rinsed with 70% ethanol and refilled with sterile water.
4. If zona-free preimplantation-stage embryos are to be cultured (for example after embryo manipulation), coat the glass-bottom dish with a small amount of 2% agarose to avoid sticking of the embryo to the dish. At E4.0 mouse embryos (corresponding to the late-stage blastocyst) begin hatching from the zona pellucida and changing their shape in the process. These changes may cause the embryo to move and obscure the visualization of processes being imaged. To overcome these movements during live imaging, the zona pellucida can be removed beforehand.

5. Successful roller culture of midgestational mouse embryos (E10.5) free of yolk sac and amnion has been reported in serum-free medium [53].
6. Embryos at E9.5 can be dissected out of their yolk sac for live imaging. Although it is feasible to culture mouse embryos in vitro beyond E10.5 [53], the size of the embryo and thickness of its tissues make imaging extremely difficult. However, recent reports using multiphoton excitation for live imaging in neonatal and adult mice [54, 55] suggest that the same technique can be used in older mouse embryos to a depth of up to 1000 μm [56].
7. For imaging at later stages, it is recommended to dissect out the region of interest (such as the ureteric buds [23] or pancreas [57]) and image as an explant culture.
8. Transferring older embryos into oil-covered medium may be a problem due to the surface tension of the medium, and a relatively large diameter of the pipette being used. To address this issue, the medium can be equilibrated prior to embryo dissection in an organ culture dish in a humidified incubator. Just before starting the culture, place a drop of the equilibrated medium in a glass-bottom dish, transfer the embryos into the drop, and cover with mineral oil.

Acknowledgments

We thank our present and past laboratory colleagues for helping perfect the techniques detailed in this chapter. Work in our laboratory is supported by the National Institutes of Health (R01DK084391, R01HD094868, and P30CA008748) and The Starr Foundation.

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Chapter 12

Detection of Gene and Protein Expression in Mouse Embryos and Tissue Sections

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Abstract

Analysis of gene (mRNA and protein) expression patterns is central to the study of embryonic development. This chapter details methods for detecting mRNA and protein expression in whole-mouse embryos and in tissue sections, including mRNA in situ hybridization, immunohistochemistry, and detection of enzymatic and fluorescent protein reporters. We focus on histological methods; molecular methods of measuring gene expression (for example, RNAseq, PCR) are not included here.

Key words Mouse embryogenesis, In situ hybridization, Immunohistochemistry, ß-Galactosidase, Alkaline phosphatase, Green fluorescent protein

1 Introduction

Most studies of embryonic development include analyses of mRNA and/or protein expression, either in unperturbed or in experimentally manipulated systems. These expression patterns can be assessed in a number of ways, depending on the organism and type of information required. For precise spatial and temporal resolution, methods for detection within embryonic tissues (in situ) are commonly used. mRNA in situ hybridization has the advantage that it can be performed for any gene with known sequence, and follows a standardized procedure. However, it is somewhat technically challenging, and mRNA expression patterns do not always reflect protein expression and localization. Immunohistochemistry has the advantage of directly detecting protein expression; however, assays must generally be optimized for each antibody, and the availability of good antibodies is a limitation. In mice, well-established methods for making transgenic and targeted knockout or knock-in animals make the detection of exogenous fluorescent or enzymatic reporter proteins another common readout of gene expression. Illustration of each of these three types of experiments is shown in Fig. 1.

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_12,

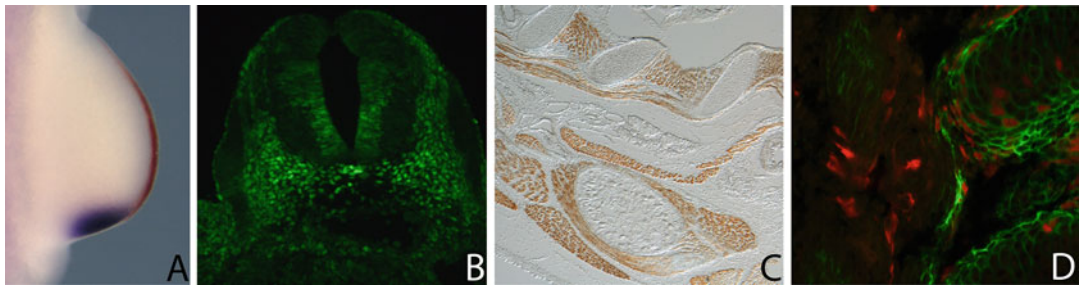

Fig. 1 (**a**) Dual-color whole-mount in situ hybridization (*see* Subheading 3.2.1). Stage 21 chick embryonic forelimb detecting two independent mRNA transcripts as follows: sonic hedgehog (arrow) using DIG-labeled riboprobe followed by NBT/BCIP color reaction; Fgf8 (arrowhead) using fluorescein-labeled riboprobe followed by INT/BCIP color reaction (see text). Reproduced with permission from [17]. (**b**) Section immunohistochemistry detected by fluorescence (*see* Subheading 3.3.2). Stage 10.5 mouse transverse section through branchial somites. IHC was used to detect the Sox9 transcription factor (primary antibody, Millipore ab 5535; secondary antibody, Jackson Immunoresearch 711-225-15). (**c**) Section immunohistochemistry detected by HRP/DAB (*see* Subheading 3.3.2). Transverse paraffin section in an E14.5 embryo reveals fast skeletal muscle myosin in the limb and intercostal muscles. This 10 μM paraffin section was processed with a monoclonal antibody (MY32, Sigma) followed by an HRP-conjugated secondary antibody and DAB detection. Antigen retrieval was performed (see text). (**d**) Direct detection of tdTomato reporter coupled with fluorescent IHC (*see* Subheadings 3.4.5 and 3.3.2). Sagittal section of E14.5 mouse embryo bearing a tdTomato reporter [18]: revealing cells labeled by activity of *14.5kbHoxa5Cre* [19]. Two adjacent neural arches are shown. Cartilage and connective tissue co-labeled with the extracellular matrix protein Tenascin (Sigma #T3413)

This chapter is divided into four sections: (1) preparation of tissue sections, (2) mRNA in situ hybridization, (3) immunohistochemistry, and (4) reporter detection. Subheadings 2–4 are further subdivided for separate whole mount and section protocols. For additional resources about these and similar methods, please *see* also [1–3]. Finally, although these protocols are written for mouse, they can be and have been adapted to other vertebrate embryos.

2 Materials

2.1 Preparation of Tissue Sections

2.1.1 Paraffin Sections

1. Phosphate-buffered saline (PBS): Prepare a 10× stock solution containing 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, and 20 mM KH_2PO_4 in dH_2O. Adjust pH to 7.4. Autoclave and store at room temperature. Make 1× PBS by diluting 1 part 10× PBS in 9 parts dH_2O. Store at room temperature.
2. Phosphate-buffered saline with Tween-20 (PBT): 0.1% Tween-20 in 1× PBS. Store at room temperature.
3. 4% Paraformaldehyde: Prepare fresh on the day of use from a 20% (w/v) stock solution of paraformaldehyde (Fisher) in PBS. To make stock solution, heat to 65–70 °C with constant mixing on a stir plate. If necessary, add a drop or two at a time of 10 N

NaOH, followed by several minutes of stirring, until the solution clears. Filter sterilize, aliquot, and store at −20 °C. Reheat stock to 65 °C to redissolve before use.

Caution: Paraformaldehyde (*see* **Note 1**).

4. Ethanol.
5. Xylene.

   Caution (*see* **Note 1**).

6. Low-melting paraffin wax, such as Paraplast X-Tra (McCormick).
7. Molds and embedding rings.
8. Superfrost Plus (Fisher) or TESPA-treated slides (*see* **Note 2**).

2.1.2 OCT Cryosections

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. 5% Sucrose in PBS (w/v): Dissolve and filter sterilize. Store at 4 °C.
3. 30% Sucrose in PBS (w/v): Dissolve and filter sterilize. Store at 4 °C.
4. OCT compound (Tissue Tek).
5. Peel-away plastic embedding molds (VWR).
6. Superfrost Plus or TESPA-treated slides (*see* **Note 2**).

2.1.3 Gelatin Cryosections

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. 4% Paraformaldehyde in PBS (Subheading 2.1.1).
3. 5% Sucrose in PBS (w/v): Dissolve and filter sterilize. Store at room temperature.
4. 20% Sucrose in PBS (w/v): Dissolve and filter sterilize. Store at room temperature.
5. Gelatin solution: 7.5% Gelatin (porcine, Sigma), 15% sucrose in PBS. To prepare: make a 15% (w/v) gelatin solution in PBS and autoclave. Make a 30% (w/v) sucrose in PBS solution and filter sterilize. Mix together in equal proportions. Aliquot and store at 4 °C. Heat to 37 °C before use.
6. Dry ice/100% ethanol bath.
7. 2-Methylbutane.

   Caution (*see* **Note 1**).

8. Superfrost Plus or TESPA-treated slides (Subheading 2.1.1).

2.2 mRNA In Situ Hybridization

2.2.1 Preparation of Riboprobes

1. DNA template corresponding to the gene of interest (*see* **Note 3**).
2. Phenol (pH 8):chloroform:isoamyl alcohol (50:49:1) can be purchased premixed or individually.

   Caution: Phenol, chloroform (*see* **Note 1**).
3. RNA polymerase (Roche T7, T3, or Sp6; 20 U/μL) with associated transcription buffer.
4. Digoxigenin (DIG) RNA Labeling Mix (Roche): For alternative labeled nucleotides used in double- and triple-in situ hybridization *see* **Note 4**.
5. RNase Inhibitor (40 U/μL, Roche).
6. RNase-free DNase (10 U/μL, Roche).
7. Diethyl pyrocarbonate (DEPC)-treated dH_2O.

   Caution: DEPC (*see* **Note 1**).
8. 3 M NaOAc (pH 5.2), made with DEPC-treated dH_2O.
9. 100% Ethanol and 70% ethanol made up by adding 7 parts ethanol to 3 parts DEPC-treated dH_2O.

2.2.2 Whole-Mount In Situ Hybridization

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. Phosphate-buffered saline with TritonX-100 (PBTX): 0.1% TritonX-100 in DEPC-treated PBS. Store at room temperature.
3. 4% Paraformaldehyde/0.2% glutaraldehyde in PBS: Prepare fresh on the day of use from 20% paraformaldehyde and 25% glutaraldehyde frozen stocks as described in Subheading 2.1.1. Glutaraldehyde can be purchased as a 25% solution.

   Caution: Paraformaldehyde, glutaraldehyde (*see* **Note 1**).
4. Methanol.

   Caution (*see* **Note 1**).
5. H_2O_2 solution: 6% H_2O_2 in PBTX (make by diluting 30% stock H_2O_2). Prepare immediately before use.
6. Proteinase K: 10 mg/mL (Roche).
7. Prehybridization/hybridization solution: 50% Formamide, 5× SSC (pH 7.0; use citric acid to pH solution), 2% blocking powder (Roche), 0.1% TritonX-100, 0.5% CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid), 1 mg/mL yeast RNA, 5 mM EDTA, and 50 mg/mL heparin made up in dH_2O. Store at −20 °C and preheat to 65 °C immediately prior to use.

   Caution: CHAPS, formamide (*see* **Note 1**).

8. Solution 1: 50% Formamide, 5× SSC, 0.5% CHAPS, and 0.1% TritonX-100 made up in dH_2O. Store at RT and preheat to 65 °C immediately prior to use.

   Caution: CHAPS, formamide (*see* **Note 1**).

9. Tris buffer with TritonX-100 (TBTX): 50 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 made up in dH_2O and store at room temperature.
10. Antibody-blocking solution: 10% Serum (Sigma) and 2% BSA in TBTX. We commonly use sheep, horse, or donkey serum. Prior to use, serum should be heat-inactivated at 70 °C for 30 min, and stored at −20 °C.
11. AP-conjugated anti-digoxigenin (DIG) antibody (Roche): For double- and triple-in situ hybridization, AP-conjugated anti-fluorescein and AP-conjugated anti-biotin antibodies are required.
12. NTMT buffer: 100 mM NaCl, 100 mM Tris–HCl pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween-20 in dH_2O. NTMT is prepared fresh each time; it will acidify over time.
13. Nitro blue tetrazolium chloride (NBT) stock: 100 mg/mL NBT (Roche) in 70% DMF (dimethyl formamide). Store at −20 °C. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock: 50 mg/mL BCIP (Roche) in dH_2O. Store at −20 °C. 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride (INT) stock: 50 mg/mL in DMSO (dimethyl sulfoxide). Store at −20 °C. Premixed substrate solution BM Purple (Roche).

2.2.3 Section In Situ Hybridization

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. Phosphate-buffered saline with 0.1% Tween-20 (PBT) (Subheading 2.1.1).
3. Hybridization solution: 10 mM Tris–HCl pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% dextran sulfate (American Bioanalytical 50% solution), 1× Denhardt's, 200 mg/mL yeast tRNA (Gibco), 50% formamide. Store at −20 °C.

   Caution: SDS, formamide (*see* **Note 1**).

4. Flexible plastic coverslips cut from polypropylene bags (BelArt).
5. SSC: 20× stock pH 7.0.
6. 10× Triethanolamine (TEA): 1 M TEA, pH 8.0.

   Caution (*see* **Note 1**).

7. Acetic anhydride.

   Caution (*see* **Note 1**).

8. TNE: 10 mM Tris–HCl pH 7.5, 500 mM NaCl, and 1 mM EDTA.
9. MAB (5×): 0.5 M Maleic acid, 0.75 M NaCl, bring to pH 7.5 with NaOH. Make 1× MABT by diluting 1 part 5× MAB in 4 parts dH_2O and add Tween-20 to a final concentration of 0.1%. Store at room temperature.
10. Antibody-blocking solution: 20% Sheep serum and 2% Boehringer Blocking Reagent (Roche) in MABT; heat to 55 °C to dissolve. Prior to use, serum should be aliquoted and stored at −20 °C.
11. NTM (pH 9.5): 100 mM NaCl, 100 mM Tris–HCl pH 9.5, and 50 mM $MgCl_2$.
12. NTM (pH 8): 100 mM NaCl, 100 mM Tris–HCl pH 8, and 50 mM $MgCl_2$.
13. Nitro blue tetrazolium chloride (NBT) stock: 100 mg/mL in 70% DMF. Store at −20 °C. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock: 50 mg/mL in dH_2O. Store at −20 °C. 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride (INT) stock: 50 mg/mL in DMSO. Store at −20 °C. Premade NBT/BCIP solution (BM Purple, Roche) and premade INT/BCIP solution (Roche) can be substituted here.
14. Fast red tablets (Sigma).
15. Gelvatol or other aqueous mounting media: Gelvatol is prepared as described in [4]: to make 1 L, dissolve 125 g PVA (AIRVOL 205 Polyvinyl Alcohol, Air Products, Allentown, PA) in 500 mL 0.14 M NaCl and 10 mM phosphate buffer pH 7.2 (50× phosphate buffer stock is made by adding 0.5 M KH_2PO_4 to 0.5 M Na_2HPO_4 until pH reaches 7.2). Stir overnight, then microwave to >90 °C (do not boil), and then continue to stir at room temperature until solution cools. Add and 500 mL glycerol, and stir until completely mixed. Allow to rest at room temperature overnight, remove any solids, then aliquot, and store at 4 °C. Heat to 65 °C before use.

2.3 Immunohistochemistry

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. Phosphate-buffered saline with 0.1% Tween-20 (PBT) (Subheading 2.1.1).
3. 4% Paraformaldehyde (Subheading 2.1.1).

   Caution: Paraformaldehyde (*see* **Note 1**).
4. Pressure cooker (Nordic Ware Tender Cooker).
5. Permeabilization materials: Citrate buffer: 1.8 mM citric acid and 8.2 mM sodium citrate dissolved in water (pH 6) prepared from from 4 M citric acid stock and powdered sodium citrate,

proteinase K (10 mg/mL stock, Roche), or 0.1–0.5% Triton-X. Prepare permeabilization solutions on the day of experiment.

6. Dent's bleach: 10% H_2O_2, 13% DMSO, in methanol. Make fresh on the day of use. H_2O_2 stock solutions (usually 30%) can be stored for several months at 4 °C, protected from light. H_2O_2 can be omitted if not detecting with a peroxidase.

   Caution: Methanol (*see* **Note 1**).

7. Blocking solution: 5% Serum (Jackson Immunoresearch), 2% Boehringer Blocking Reagent (Roche) in PBT. We prepare 10% BBR/PBS stocks by heating to approximately 70 °C to dissolve and storing at −20 °C. Ideally, serum should be from the same species that the secondary antibody was raised in. Optional: 0.1–0.5% Triton-X or 20% DMSO can be added to blocking solution to increase permeability. For mouse-on-mouse (MOM) IHC, anti-mouse IgG Fab fragment (Jackson Immunoresearch, 115-007-003) should be added to the blocking solution at a dilution of 1:10.

8. Primary antibody of interest: See IHC methods introduction for guidance.

9. Secondary antibody against species primary antibody was raised in Jackson Immunoresearch. Use HRP-conjugated secondary antibody for whole-mount and fluorophore-conjugated secondary antibody for section IHC. We use Alexa Fluor and cyanine dyes. Alternative methods, using AP-conjugated secondary antibodies, are described in Subheading 3.3.1.

10. DAB solution: 1 mg/mL Diaminobenzidine in 100 mM Tris–HCl pH 7.2. We use DAB tablets (Sigma), which eliminate the need for weighing out the highly toxic powder. Tablet should be dissolved immediately before use.

    Caution: Diaminobenzidine (*see* **Note 1**).

11. H_2O_2 solution: 0.04% H_2O_2 in water (make by diluting 30% stock H_2O_2). Prepare immediately before use.

12. Detection buffer: Immediately before use, mix equal parts DAB solution and H_2O_2 solution.

13. BABB: Mix one part benzyl alcohol with two parts benzyl benzoate. Store in a fume hood at room temperature (for whole mount only).

    Caution: Benzyl alcohol, benzyl benzoate (**Note 1**).

14. DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride), Sigma.

15. Slide staining tray (*see* **Note 5**, for sections only).

16. Pap (hydrophobic) pen (Invitrogen; for sections only).

17. Gelvatol or other aqueous mountant (for sections only; gelvatol preparation described in Subheading 2.2.3): We use antifade mountant ProLong Diamond (Invitrogen) for fluorescently labeled sections.

2.4 Reporter Detection

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. Phosphate-buffered saline with 0.1% Tween-20 (PBT) (Subheading 2.1.1).

2.4.1 ß-Galactosidase Detection

3. Fixation buffer: 2% Paraformaldehyde, 0.2% glutaraldehyde in 1× PBS. Make fresh on the day of use from 20% paraformaldehyde and 25% glutaraldehyde frozen stocks. Caution: Paraformaldehyde, glutaraldehyde (*see* **Note 1**).
4. 1 M Sodium phosphate buffer (NaP buffer): Add 1 M NaH_2PO_4 (pH 4.0) stock solution to 1 M Na_2HPO_4 (pH 8.8) stock solution until the solution reaches pH 7.4 (approximately 150 mL NaH_2PO_4 per L Na_2HPO_4 (pH 8.8)). Autoclave and store at room temperature.
5. ß-gal Washing buffer: 100 mM NaP buffer, 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% Nonidet P-20. Filter-sterilize and store at room temperature.
6. ß-gal Detection buffer: 5 mM $K_3Fe(CN)6$, 5 mM $K_4Fe(CN)6$, 1 mg/mL X-gal in ß-gal washing buffer. Make immediately before use from stock solutions of $K_3Fe(CN)6$ and $K_4Fe(CN)6$ (each solution is 0.5 M, in dH_2O, stored at room temperature and protected from light).
7. Gelvatol (for sections only; preparation described in Subheading 2.2.3).

2.4.2 Alkaline Phosphatase Detection

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. Phosphate-buffered saline with 0.1% Tween-20 (PBT) (Subheading 2.1.1).
3. 4% Paraformaldehyde in PBS (Subheading 2.1.1).

   Caution: Paraformaldehyde (*see* **Note 1**).
4. NTM buffer: 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. Optional: 0.1% Tween-20; Tween will increase the reaction rate, but also the rate of background color development. Prepare on the day of use; NTM will acidify over time.
5. AP detection buffer: Immediately before use, add to NTM (or NTMT) buffer: 4-Nitro blue tetrazolium chloride (NBT, Roche) to a final concentration of 0.45 mg/mL and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP, Roche), to a final concentration of 0.175 mg/mL.
6. Gelvatol (for sections only; preparation described in Subheading 2.2.3).

2.4.3 Fluorescent Reporter Detection

1. Phosphate-buffered saline (PBS) (Subheading 2.1.1).
2. 4% Paraformaldehyde in PBS (Subheading 2.1.1).

   Caution: Paraformaldehyde (*see* **Note 1**).
3. Gelvatol (for sections only; preparation described in Subheading 2.2.3) or anti-fade aqueous mountant such as Prolong Diamond (Invitrogen).

3 Methods

3.1 Preparation of Tissue Sections

To achieve cellular resolution of gene expression with any of the following techniques, tissues can be embedded in paraffin or cryoembedded in OCT or gelatin and sectioned. Paraffin sections produce the best morphology and adherence of tissue to slides, while OCT cryosections produce the highest signal. Paraffin embedding destroys some antigens and thus cannot be used with some antibodies. Gelatin cryosections produce relatively good morphology compared to OCT, and are recommended for sectioning tissues stained prior to sectioning in whole mount. The embedding protocols detailed here are courtesy of Constance Cepko and Clifford Tabin (Harvard Medical School, Boston, MA, USA).

3.1.1 Paraffin Sections

1. Dissect embryos out in ice-cold PBS and transfer to glass vials (*see* **Note 6**).
2. Incubate in 4% paraformaldehyde at 4 °C, rocking, for 1 h to overnight (*see* **Note 7**).

   For younger embryos (<E12.5) 1–2 h at 4 °C is recommended; for older embryos a longer fix may be needed. Overfixation causes brittle tissue and crumpled sections, and can mask epitopes for IHC.
3. Wash embryos twice in PBS.
4. Dehydrate embryos through a graded ethanol series with 10-min washes rocking at room temperature, as follows:

   25% Ethanol/PBS.

   50% Ethanol/PBS.

   75% Ethanol/dH_2O.

   100% Ethanol.

   100% Ethanol.

   For older embryos (>E12.5), increased wash times and additional incremental increases between 75 and 100% are beneficial for preserving morphology.
5. Optional: Embryos may be stored at −20 °C in 100% ethanol at this stage for several months at least.

6. Clear embryos by incubating in xylene twice for 5–30 min (depending on the embryo size), rocking at room temperature. Both over- and undertreatment with xylene may lead to brittle, crumpled sections.
7. Incubate embryos in 50:50 xylene:paraffin for 15 min at 60 °C.
8. Incubate embryos in 100% paraffin 3–5 times for 1 h at 60 °C.
9. Incubate embryos in paraffin, at 60 °C, under a vacuum, for at least 1 h.

   Steps 8 and **9** can be extended overnight if necessary; however if problems with in situ signal strength are observed then strict adherence to same-day processing may be helpful.
10. Embed embryos and store blocks at 4 °C until needed.
11. Section and collect on Superfrost Plus or TESPA-treated slides (*see* **Note 2**).
12. Dry sections overnight at 37 °C and store at 4 °C until use.

3.1.2 OCT Cryosections

1. Dissect embryos out in ice-cold PBS and transfer to glass vials or plastic 6-well plates (*see* **Note 6**).
2. Incubate embryos in 4% paraformaldehyde for 1 h to overnight, at 4 °C. Shorter fixation (1–2 h) is preferred for embryos <E12.5, but longer may be necessary for older embryos. Overfixation can mask epitopes. *See* also **Note 7**.
3. Wash embryos in PBS 3× for 5 min.
4. Transfer embryos to 5% sucrose/PBS, and rock at 4 °C for several hours, until embryos sink.
5. Incubate embryos in 20% sucrose/PBS, rocking overnight at 4 °C.
6. Prepare 50:50 solution of 20% sucrose/PBS:OCT and rock solution to homogenize for 10 min. To avoid bubbles, do not vortex. Incubate embryos in solution, rocking for 2–3 h at 4 °C.
7. Rock embryos in 100% OCT for 2 h–overnight at 4 °C.
8. Embed embryos in OCT, in peel-away plastic molds, on dry ice and store blocks at −80 °C in freezer bag.
9. Section and collect on either Superfrost Plus or TESPA-treated slides.
10. Air-dry sections by placing on 37 °C plate for 20 min and store sections at −80 °C.

3.1.3 Gelatin Cryosections

1. Dissect embryos or tissues into ice-cold PBS and transfer to glass vials (*see* **Note 6**).
2. Incubate in 4% paraformaldehyde several hours to overnight (*see* **Note 7**).

If the tissue has already been processed in whole mount, skip **steps 1** and **2**.

3. Wash embryos in PBS, once quickly, and then 3× 5 min rocking, at room temperature.
4. Incubate embryos in 5% sucrose/PBS until embryos equilibrate (until they sink, rocking at 4 °C).
5. Incubate embryos in 20% sucrose/PBS until embryos equilibrate, rocking at 4 °C.
6. Incubate in pre-warmed gelatin solution at 37 °C for 1.5–4 h (depending on the stage of the embryo).
7. Change to fresh gelatin solution and embed embryos in peel-away plastic molds (VWR). Place molds on a flat layer of wet ice and position embryos under a dissecting microscope. It is helpful to allow a thin layer of gelatin to partially solidify in the bottom of the mold before adding the embryo, so that the embryo will not be right at the surface of the block. However, if this layer becomes too solid, then the block can crack at the interface during freezing.
8. Leave blocks on ice until gelatin is set, but do not allow to freeze.
9. Store blocks at 4 °C, in a humidified box, to completely set the gelatin (overnight to 2 days).
10. Prepare a dry ice/ethanol bath, place a beaker of 2-methylbutane in the bath, and allow to cool.
11. Peel away plastic molds. Cut blocks to the size with a razor blade. Make handles for freezing by folding strips of cardboard into an L shape. Place gelatin block in the bottom of the L with the cutting surface up.
12. Lower blocks on cardboard handles into the 2-methylbutane. Immerse until frozen (around 5 s) but do not over-freeze, as blocks may crack.
13. Store blocks at −80 °C until use.
14. Section blocks on a cryostat, collect on Superfrost Plus or TESPA-treated slides (*see* **Note 2**), and air-dry briefly.
15. Store slides at −80 °C until use.

3.2 RNA In Situ Hybridization

The ability to detect RNA transcripts within the developing embryo relies on a stable hybridization reaction between a labeled antisense RNA probe (riboprobe) and the RNA transcript of interest. To interrogate gene expression within the context of the entire embryo, whole-mount in situ hybridization is performed, and the protocol detailed here is based on early methods [5] with slight modifications [6]. Multiple RNA transcripts can be detected simultaneously, as shown in Fig. 1a. To obtain cellular resolution of gene

expression, section in situ hybridization is performed, and the protocol detailed here is courtesy of Constance Cepko and Clifford Tabin (Harvard Medical School, Boston, MA, USA). Both protocols utilize colorimetric detection of the riboprobe; however it can be substituted with fluorescent detection if required. The detection of small RNA species such as mature microRNAs requires protocol optimization, particularly at the level of tissue fixation, probe design, and hybridization temperature. These alterations to core in situ protocols are beyond the scope of this chapter; however useful references include [7–9]. Before embarking on this technically challenging procedure, it is recommended to search various publically available gene expression databases in which a vast number of developmental expression patterns are cataloged. Such databases include EMAGE (http://www.emouseatlas.org/emage/), MGI (http://www.informatics.jax.org/), and Genepaint (http://www.genepaint.org/).

3.2.1 Riboprobe Generation

Considerations for choice of riboprobe template sequence are discussed (*see* **Note 3**). Generation of DNA template for riboprobe synthesis can be achieved by either plasmid linearization or PCR amplification.

1. For template generation by plasmid linearization (**steps 1–13**), linearize 1 μg of plasmid with an appropriate enzyme, preferably to give a 5′ overhang (if a 3′ overhang is generated, blunt using the 3′ exonuclease activity of Klenow). For considerations related to labeling choices when using multiple probes *see* **Note 4**.
2. Run 50 ng on a 1% agarose gel to check digest is complete.

   Important: All steps from now on should be carried out in RNase-free (RF) conditions (*see* **Note 8**).
3. Dilute to 200–500 μL with DEPC-treated dH_2O.
4. Add an equal volume of phenol (pH 8):chloroform:isoamyl alcohol (50:49:1), vortex, and centrifuge for 2 min.
5. Remove upper layer and place in a new tube.
6. Add an equal volume of chloroform:isoamyl alcohol (49:1), vortex, and centrifuge for 2 min.
7. Remove upper layer and place in a new tube.
8. Add 1/10 volume of RF 3 M NaOAc (pH 5.2), and 2.5 volumes of cold RF 100% ethanol.
9. Precipitate on ice for 15 min.
10. Spin down in microfuge for 15 min at maximum speed at 4 °C.
11. Wash pellet once with cold RF 70% ethanol and air-dry for 10 min.
12. Resuspend in 11.5 μL DEPC H_2O.

13. Run 1 μL on a 1% agarose gel to check template before proceeding to the transcription reaction.
14. **Steps 14–18** provide an alternative approach to generate template through PCR amplification. Using 10 ng plasmid DNA as a template, amplify insert sequence by PCR.

    PCR primer sequences must be either external to or directly corresponding to the RNA polymerase-binding sites.
15. Run 1 μL on a 1% agarose gel to check single PCR product generation.
16. Add DEPC H_2O to a final volume of 50 μL.
17. Add 50 μL chloroform, mix, and centrifuge at maximum speed for 2 min at room temperature.
18. Remove upper layer (approximately 40 μL) and place in a new tube. In general, 2–4 μL of purified PCR product will be sufficient for a transcription reaction.
19. Once the DNA template has been generated, proceed to riboprobe transcription (**steps 19–27**). Add reagents to Eppendorf tubes in the following order:

    2 μL 10× transcription buffer

    2 μL Digoxigenin (DIG) RNA labeling mix

    x μL DNA template (approximately 1 μg)

    0.5 μL Ribonuclease inhibitor (40 U/μL)

    2 μL SP6, T7, or T3 RNA polymerase (10 U/μL)

    DEPC-treated dH_2O to 20 μL
20. Incubate at 37 °C (or 40 °C if using SP6) for 2 h.
21. Remove a 1 μL aliquot and run on 1% agarose gel to check synthesis (*see* **Note 9**).
22. Optional: Add 1 μL DNase (10 U/μL, RNase free, Roche) and incubate at 37 °C for 15 min.
23. Dilute the probe to 50 μL with DEPC H_2O, and add 1/10 volume of RF 3 M NaOAc (pH 5.2), and 2.5 volumes of RF 100% ethanol.

    If probe yield is low, addition of 1 μL of RF glycogen (20 μg/μL) during precipitation is helpful.
24. Precipitate on ice for 30 min.
25. Centrifuge at maximum speed for 20 min at 4 °C.
26. Wash pellet once with RNase-free 70% ethanol, air-dry, and resuspend in 100 μL DEPC-treated dH_2O.
27. Run 5 μL on a 1% agarose gel to check probe; approximate probe concentration can be estimated by comparison to a standard DNA ladder (*see* **Note 9**).

3.2.2 Whole-Mount RNA In Situ Hybridization

Important: All steps prior to and including hybridization (**steps 1–17**) should be carried out in RNase-free (RF) conditions (*see* **Note 8**).

1. Dissect embryos out in ice-cold DEPC-treated phosphate-buffered saline (PBS). To prevent probe trapping, puncture the neural tube once it has closed (E9.5 onwards). If dissecting out small embryos (E9.0 or younger), use PBS containing 100 mg/L $MgCl_2$ and 100 mg/L $CaCl_2$ to maintain tissue integrity (for example: Dulbecco's modified PBS, Sigma). Transfer to glass vials (*see* **Note 6**).
2. Incubate embryos in 4% paraformaldehyde at 4 °C, rocking, overnight. For younger embryos (9.5 dpc and younger) this step should be shortened to 2 h at 4 °C.
3. Wash 2× for 10 min with PBTX at 4 °C, rocking.
4. Dehydrate embryos through a graded methanol/PBS series with 20-min washes rocking at room temperature, as follows:

   25% Methanol/PBS

   50% Methanol/PBS

   75% Methanol/PBS

   100% Methanol

   100% Methanol
5. Optional: Embryos may be stored at −20 °C in 100% methanol at this stage for several months at least.
6. Optional: Bleach embryos with 6% H_2O_2 in methanol for 1 h at RT with gentle rocking.
7. Rehydrate embryos through a graded methanol/PBS series with 20-min washes rocking at room temperature, as follows:

   75% Methanol/PBS

   50% Methanol/PBS

   25% Methanol/PBS
8. Wash 3× for 10 min in PBTX.
9. Treat with 10 μg/mL proteinase K in PBTX for 2–35 min depending on the stage of embryo.

   Each new batch of proteinase K may need to be tested for appropriate treatment times. For consistent results proteinase K stock (10 mg/mL) should be distributed into single-use aliquots and stored at −20 °C. Make a fresh dilution to 10 μg/mL when treating embryos.

Approximate treatment times are as follows:

7.5 dpc	2 min
8.5 dpc	5 min
9.5 dpc	10 min
10.5 dpc	15 min
11.5 dpc	20 min
12.5 dpc	25 min
13.5 dpc	30 min
14.5 dpc	35 min

10. Wash 2× for 5 min with PBTX.

    Take care with washes—the embryos are fragile.
11. Refix with 0.2% glutaraldehyde/4% paraformaldehyde in PBTX with gentle rocking at room temperature for 20 min.
12. Wash 2× for 10 min with PBTX.
13. Transfer embryos to hybridization vials (*see* **Note 8**). Remove as much PBTX as possible and add enough prehybridization solution to completely cover the embryos. Allow embryos to sink to the bottom.
14. Incubate at 65 °C for 2 h to overnight with constant agitations (*see* **Note 10**). Prehybridization time can be extended.
15. Remove liquid and replace with a fresh volume of prehybridization solution. We commonly do not change the prehybridization solution but ensure that minimal amounts of PBTX are carried over when embryos are transferred to the prehybridization solution.
16. Add probe (0.2–1.0 μg/mL DIG-labeled probe, determined empirically) to prehybridization solution.
17. Incubate overnight at 65 °C. Hybridization temperature can be altered (*see* **Note 11**).
18. At this point RNase-free conditions are no longer necessary. Wash embryos with constant agitation for 5 min each at 65 °C as follows:

    100% Solution 1

    75% Solution 1/25% 2× SSC

    50% Solution 1/50% 2× SSC

    25% Solution 1/75% 2× SSC

    These washes can be done in a heating block on a rocker or in hybaid bottles (*see* **Note 10**).

19. Wash two times with 2× SSC and 0.1% CHAPS at 55–65 °C, 10 min each wash.
20. Wash two times with 0.2× SSC and 0.1% CHAPS at 55–65 °C, 15 min each wash.
21. Wash two times with TBTX at RT, 10 min each wash.
22. Incubate embryos with antibody-blocking solution (freshly made) for 1 h rocking at room temperature.
23. Optional: Preadsorb anti-DIG antibody to remove nonspecific antibodies (*see* **Note 12**). We routinely skip this step because the quality of commercial DIG antibodies is high.
24. Remove blocking solution and replace with blocking solution plus anti-DIG antibody diluted at 1:2000.
25. Rock overnight at 4 °C. Antibody incubation can be shortened to 4 h at room temperature.
26. Remove antibody solution. Antibody solution can be reused a number of times for up to a month as long as it is stored at 4 °C and no growth is observed in the high-serum solution.
27. Wash five times with TBTX plus 0.1% BSA at room temperature, 30 min each wash.
28. Wash overnight with TBTX plus 0.1% BSA at 4 °C.
29. To begin histochemical detection, wash two times in TBTX at RT for 15 min each wash.
30. Wash three times in fresh NTMT for 10 min each wash.
31. Remove NTMT wash and add NTMT including 3.5 μL of 100 mg/mL NBT and 3.5 μL 50 mg/mL BCIP per mL. Alternatively, use of the premixed substrate solution BM Purple is highly recommended as background staining is significantly reduced.
32. Keep embryos in the dark as much as possible and rock gently until the color has developed to the desired extent.
33. Wash with NTMT (5–10 min, do not leave too long) and then PBTX for 20 min. If color has not developed before it is time to leave, the embryos can be washed in NTMT and then TBTX, stored at 4 °C overnight, and the color development restarted at **step 2** the next day.
34. Wash several times in PBS plus 1% Triton X-100. The last wash can be done overnight at 4 °C—this helps remove background.
35. Fix stained embryos in 4% paraformaldehyde in PBTX overnight at 4 °C.
36. Exchange embryos into PBS and photograph as soon as possible (*see* **Note 13** for photography suggestions). To store for extended periods embryos can be kept at 4 °C in PBS + sodium azide, or can be taken through a PBTX series into 100% glycerol.

3.2.3 Section RNA In Situ Hybridization

Embryonic samples can be processed by either OCT cryosection or paraffin section prior to RNA in situ hybridization analysis (Subheadings 3.1.1 and 3.1.2). Signal strength is often greater following cryosection while preservation of tissue morphology is often better following paraffin section. Generate riboprobe as in Subheading 3.2.1 and first prepare slides for hybridization (paraffin sections, **steps 1–4**; frozen sections, **step 5**).

Important: All steps prior to and including hybridization should be carried out in RNase-free (RF) conditions (*see* **Note 8**).

1. To prepare slides for paraffin sections (**steps 1–4**), first bake slides on hot plate at 60 °C for 1 h.
2. Allow slides to come to room temperature.
3. Dewax in xylene 2× for 5 min.
4. Rehydrate sections through a graded ethanol series with 5-min washes at room temperature, as follows:

   100% Ethanol

   100% Ethanol

   75% Ethanol/dH_2O

   50% Ethanol/PBS

   25% Ethanol/PBS

   PBS

   PBS
5. To prepare slides for frozen sections, thaw slides and air-dry for 15–20 min at room temperature.
6. Incubate slides in 4% paraformaldehyde for 10 min.
7. Rinse briefly and then wash twice for 5 min in PBT.
8. Treat slides with proteinase K diluted in PBS (generally 1 μg/mL for 10 min, but concentration and length of incubation can be varied).
9. Wash twice in PBT for 5 min.
10. Incubate slides in 4% paraformaldehyde for 5 min. PFA from first post-fix can be reused.
11. Rinse briefly and then wash twice for 5 min in PBT.
12. Incubate slide for 10 min in acetylation reaction mix. To prepare acetylation mix—add 625 μL acetic anhydride to 250 mL 0.1 M TEA (triethanolamine) in a fume hood and shake well; use immediately.
13. Rinse briefly and then wash twice for 5 min in PBT.
14. Rinse briefly in dH_2O.
15. Blot excess liquid and air-dry slides for no more than 10 min, until any remaining droplets have evaporated, but do not allow

tissue to become completely dry. Process 5–10 slides at a time so slides do not overdry.

16. Combine 100 μL prewarmed hybridization solution at 65 °C and 1 μL of probe, add carefully to slides to avoid air bubbles, and add coverslip. For double- or triple-in situ hybridizations, add 1 μL of each probe to 100 μL hybridization solution.
17. Place slides in humidified slide box (use Whatman paper or paper towels soaked in 5× SSC/50% formamide).

    Alternatively, slides can be completely submerged in a slide mailer box (four-slide box) containing 8 mL hybridization solution plus riboprobe. This solution can be reused multiple times if stored at −20 °C.
18. Incubate overnight at 65 °C.
19. Important: Pre-warm all post-hybridization washes (**steps 18–25**). Remove coverslips by allowing them to float off (do not pull) in 5× SSC at 65 °C.
20. Wash slides in 1× SSC/50% formamide for 30 min at 65 °C.
21. Wash slides in TNE for 10 min at 37 °C.
22. Incubate slides in TNE plus RNase A (20 μg/mL, Roche) for 30 min at 37 °C.
23. Wash slides in TNE for 10 min at 37 °C. Reuse TNE from first wash.
24. Wash slides in 2× SSC for 20 min at 65 °C.
25. Wash slides in 0.2× SSC for 20 min at 65 °C.
26. Wash slides in 0.2× SSC for 20 min at 65 °C.
27. Proceed to antibody incubation and histochemical detection (**steps 26–55**). First, wash slides in MABT twice for 5 min at RT.
28. Incubate in antibody blocking solution for a minimum of 1 h.
29. Add anti-DIG-AP antibody (Roche), diluted 1:2000 in 2% sheep serum/MABT. Add 500 μL - 1 mL per slide and incubate overnight at 4 °C in humidified chamber (*see* **Note 5**). Antibody incubation can be reduced to 3 h at room temperature.
30. Rinse briefly in MABT.
31. Wash three times in MABT for 5 min.
32. Wash slides in NTM pH 9.5 for 10 min. Tween is not generally added to NTM for section in situ hybridization. Tween will speed up the reaction but causes higher background levels.
33. Incubate slides with 1 mL NTM pH 9.5 with 4.5 μL NBT (50 mg/mL) and 7.0 μL BCIP (25 mg/mL).
34. Allow color to develop for 1 h to 3 days in the dark depending on the signal intensity. Changing the solution frequently

reduces background—change twice daily, or as soon as detection solution becomes brown.

35. Rinse briefly in NTM pH 9.5.
36. Wash slides in PBS twice for 5 min.
37. Fix color by incubating slides in 4% paraformaldehyde for 30 min. Fix for 10 min if doing double or triple detection.
38. Wash twice in PBS for 5 min.
39. For single detection: rinse slides in dH_2O and mount in gelvatol. Heat gelvatol to 65 °C before using.

    Proceed to **steps 40–47** for detection of a second probe.

40. Wash twice with MABT for 5 min.
41. Incubate in antibody-blocking solution for a minimum of 1 h.
42. Add anti-FITC-AP antibody (Roche), diluted 1:2500 in 5% sheep serum/MABT. Add 1 mL per slide and incubate overnight at 4 °C in humidified chamber.
43. Rinse briefly in MABT.
44. Wash three times for 5 min with MABT.
45. Wash slides in NTM pH 9.5 for 10 min.
46. Incubate slides with NTM pH 9.5 + INT + BCIP.
47. Allow color to develop for 1 h to 3 days (depending on the signal intensity) in the dark. Change solution as necessary.

    For double detection wash and mount slides as detailed in Subheading 3.2.3, **steps 36–39**.

    For triple detection: go on to Subheading 3.2.3, **step 48**.

48. For detection of a third probe, first wash twice with MABT for 5 min.
49. Incubate in antibody-blocking solution for a minimum of 1 h.
50. Add anti-biotin-AP antibody (Roche), diluted 1:2500 in 5% sheep serum/MABT. Add 1 mL per slide and incubate overnight at 4 °C in humidified chamber.
51. Rinse briefly in MABT.
52. Wash three times for 5 min with MABT.
53. Wash slides in NTM pH 8.0 for 10 min.
54. Dissolve Sigma Fast Red TR/Naphtali AS-MX ready-made tablets (Sigma) in buffer provided and filter through 0.2–0.8 mm syringe filter. Dilute 1:10 in NTM pH 8.0 and add to slides.
55. Develop for 1 h to 3 days (depending on the signal intensity) in the dark. Change solution as necessary.
56. Rinse briefly in NTM pH 8.0. Wash and mount slides as detailed in **steps 36–39**.

3.3 Immunohistochemistry

Immunohistochemistry protocols must be optimized for each antibody, primarily because the stability and accessibility of different epitopes are variable. Some are sensitive to heat or organic solvents, and aldehyde-induced protein cross-linking can block an antibody's access to its epitope. In this case, epitopes must be unmasked by an antigen retrieval/permeabilization step after fixation. Many methods are available, and the best for a given antibody should be determined by consulting the primary literature for established protocols, and ultimately by empirical testing. Although many commercial antibodies are available, quality is highly variable. If an antibody fails to perform after many attempts, it is likely that the antibody does not function as promised, and alternatives should be considered. CiteAb, an online database that ranks antibodies by the quantity of their citations, can be used to find quality commercial antibodies [10]. Finally, different fixation and antigen retrieval conditions can affect the subcellular distribution of antigens differently. For a full discussion of these issues, please *see* [11]. *See* also [1] for a complete discussion of antibodies and antibody-related techniques.

The whole-mount protocol, based on [12], uses an HRP-conjugated secondary antibody and DAB detection, which gives strong signal and unlike AP does not require heating (Fig. 1c shows a section IHC detected with DAB). For section IHC, the protocol usually recommended and described here uses fluorophore-conjugated secondary antibodies (Fig. 1b, d), which have the advantage of using multiple labels simultaneously. Notes for modifying these protocols to detect with alkaline phosphatase or HRP are referenced throughout (and *see* **Note 14**).

3.3.1 Whole-Mount Immunohistochemistry

1. Dissect embryos or tissues into ice-cold PBS. For older embryos, it may be necessary to dissect away individual organs, or to bisect the embryo for better antibody penetration. For stages after the neural tube has closed, puncture the neural tube to prevent trapping of reagents. Transfer to glass vials (**Note 6**).
2. Incubate embryos in 4% paraformaldehyde at 4 °C, rocking, overnight. For younger embryos (<E12.5) this step can be shortened to 1–2 h (*see* **Note 15**).
3. Wash embryos in PBT quickly, and then 3× for 10 min
4. Fix and permeabilize embryos in Dent's bleach overnight, 4 °C rocking. This step can sometimes be skipped for smaller embryos and tissues, depending on the antibody (and *see* **Notes 16** and **17**).
5. Optional: Embryos may be transferred to 100% methanol and stored at −20 °C.

6. Rehydrate embryos through a graded methanol/PBS series with 10-min washes, as follows:

   75% Methanol/dH_2O

   50% Methanol/PBS

   25% Methanol/PBS

7. Wash embryos in PBT quickly, and then 2× for 10 min (*see* **Note 18** for additional step if using alkaline phosphatase detection).
8. Incubate embryos in blocking solution for 1-several hours, rocking. Embryos may be left overnight in blocking reagent at 4 °C. If background is a problem, extending blocking time to at least overnight can be helpful.
9. Incubate embryos in primary antibody, diluted in blocking solution, at 4 °C overnight, rocking. Antibody dilution must be determined empirically. *See* also **Note 19**.
10. Wash embryos in PBT 3× for 10 min, and then 5× for 1 h.
11. Incubate embryos in secondary antibody, diluted in blocking solution, at 4 °C overnight. The optimal dilution should be determined empirically but for many commercially available secondary antibodies (such as from Jackson Immunoresearch), 1:500–1:1000 is a good starting point.
12. Wash embryos in PBT 3× for 10 min, and then 5× for 1 h. If using a fluorophore-conjugated secondary antibody, proceed to **step 15**. If using alkaline phosphatase detection, proceed as described in Subheading 3.4.3, **steps 5–10**.
13. Incubate embryos in HRP detection buffer, observing embryos closely for color development. This can begin within seconds, but can also take minutes to hours. Embryos should be monitored to determine reaction speed. *See* also **Note 20**.
14. Wash embryos in PBT, once quickly, and then 3× for 10 min.
15. Post-fix embryos in 4% paraformaldehyde, 30 min to overnight.
16. Wash embryos in PBT, once quickly, and then 3× for 10 min.
17. Optional: If clearing is not required, proceed directly to photography.
18. To clear, dehydrate embryos through a methanol/PBS series, incubating 10 min in each of the following solutions:

    25% Methanol/PBS

    50% Methanol/PBS

    75% Methanol/PBS

    2× 100% Methanol

19. Incubate in 50% methanol and 50% BABB for 5 min.
20. Incubate in BABB, in a glass container, until clear (*see* **Note 21** for considerations and optional clearing protocol).
21. Photograph under a dissecting microscope (*see* **Note 13**). Signal will fade over time, so it is best to photograph immediately.

3.3.2 Section Immunohistochemistry

1. Prepare paraffin or OCT cryosections as described in Subheadings 3.1.1 and 3.1.2, respectively. Many antigens do not survive the paraffin embedding process (*see* **Note 22** for sectioning considerations). To modify the protocol for AP or HRP detection, *see* **Notes 18** and **23**.
2. For cryosections only: thaw slides and air-dry for 15–20 min at room temperature. If endogenous fluorophores are present, prevent exposure to light. For paraffin sections, follow Subheading 3.2.3, **steps 1–4**, to dewax and rehydrate.
3. Wash slides briefly in PBS.
4. Optional: Expose the epitope using one of the methods listed below. *See* **Note 24** for advantages and drawbacks to each method. Epitope exposure can be performed by antigen retrieval (*see* **Note 25**), Triton-X permeabilization (*see* **Note 26**), or enzymatic digestion (*see* **Note 27**).
5. Using a pap pen, draw a line across the right edge of the slide label, to prevent fluid from covering the label. Blot slides and lay them flat inside a humidified staining tray.
6. Gently pipette 1 mL of blocking solution onto each slide, taking care to avoid pipetting directly onto tissue. Cover humidified tray and incubate for 1 h or more at room temperature. If background is a problem, increase block time to overnight at 4 °C.
7. For MOM only, incubate slides overnight at 4 ° C in anti-mouse IgG fab fragment diluted in blocking solution (*see* **Note 28** for additional information).
8. Remove blocking solution, and incubate slides in 1° antibody diluted in blocking solution overnight at 4 °C in covered, humidified tray (*see* **Notes 5** and **19**). To preserve antibody, prepare 100 μL solution/slide, adding flexible plastic coverslips to prevent dehydration.
9. Return slides to slide staining bucket, and allow coverslips to gently float off in PBS. Do not pull coverslips off.
10. Wash slides in PBT once quickly, and then 5× for 5 min.
11. Blot slides and lay flat on a staining tray. Incubate in 1 mL 2° antibody, diluted in blocking solution for 1–3 h at room temperature or overnight at 4 °C in covered, humidified tray. Protect slides from light for remainder of procedure.

12. Return slides to buckets and wash in PBT once quickly, and then 5× for 5 min. Optional: Stain nuclei by adding 10 μg/mL DAPI to the first of these washes.
13. Post-fix in 4% paraformaldehyde for 5–30 min. This step is not necessary if slides will be immediately imaged. Wash slides in PBT, once quickly, and then three times for 5 min.
14. Mount slides in gelvatol (preheated to 65 ° C) or anti-fade mounting medium by pipetting 1–2 drops of solution onto slides. Add glass coverslip to each, being careful to avoid bubbles. Drain excess media by placing slides in a slide rack lined with paper towel overnight at room temperature, taking care to protect from light.

3.4 Reporter Detection

A major strength of the mouse system is its genetics, including the ability to detect gene expression using reporter proteins in transgenic or knock-in animals. Reporter detection is generally easier than the methods above, because they are stable proteins with robust detection assays, and are already present in the tissue, so permeabilization is unnecessary.

We describe direct detection of fluorescent protein (FP), ß-galactosidase, and alkaline phosphatase reporters. An enormous advantage of FPs (which include GFP and its variants) is that they can be directly visualized in living tissue. Many FP variants exist, and they range widely in stability, brightness, and sensitivity to fixation and pH. The RFP variant tdTomato is a particularly bright and highly stable reporter (Fig. 1d). TdTomato can be visualized in live tissue and shows little sensitivity to fixation with paraformaldehyde, and in fact must be fixed with PFA if endogenous signal is to be preserved for IHC. In contrast, GFP and YFP signals require PFA fixation for retention but this also weakens signal, so we routinely detect them by IHC rather than directly (*see* **Note 31**). In the absence of fixation, and depending on their subcellular localization, FPs can escape from the tissue during sectioning and/or permeabilization resulting in signal loss. Note that antigen retrieval and some other techniques used to co-stain tissues denature endogenous FPs, and necessitate detection by IHC. For a more complete discussion of FP variants, please *see* [13].

ß-Galactosidase and alkaline phosphatase are both very stable enzymatic reporters that produce strong signal and low background in most mouse tissues (*see* **Notes 29** and **30** and [2, 14] for further discussion). An example of a ß-galactosidase detection is shown in Fig. 1c. ß-Galactosidase activity is more sensitive to heat, fixation, and organic solvents than is alkaline phosphatase. Both are generally used in colorimetric assays, but fluorescent detection methods are available. Further, FPs and ß-galactosidase are available in many forms, including fusions and tags that target them to particular subcellular regions and destabilized variants with short

half-lives that serve as better markers for active transcription. The following are general protocols for detecting these three reporters in whole or sectioned mouse tissues, courtesy of Constance Cepko and Clifford Tabin (Harvard Medical School, Boston, MA, USA).

3.4.1 ß-Galactosidase Detection in Whole Mount

1. Dissect embryos or tissues into ice-cold PBS. For older embryos (>E12.5) it may be helpful to bisect or dissect out tissues of interest in order to keep fixation times to a minimum, as long fixes decrease enzymatic activity (*see* **Note 29**). Transfer to glass vials (*see* **Note 6**).
2. Incubate in fixation buffer, rocking, at 4 °C:

   For embryos E9.5-E12.5, 30 min

   For younger embryos, fix 15 min

   For older embryos, fixation time can be increased to up 2 h [15]
3. Wash embryos in ß-galactosidase wash buffer once quickly, and then 3× for 15 min at room temperature.
4. Incubate embryos in ß-gal staining buffer at 37 °C, protected from light, for several hours-overnight. Observe embryos to determine the rate of reaction.
5. Wash embryos in PBS once quickly, and then 3× for 15 min. For embryos older than E12.5, more washes may be required; 15-min washes should be continued until the wash solution remains clear, not yellow.
6. Post-fix embryos in 4% paraformaldehyde in PBS, 30 min to overnight, rocking.
7. Wash embryos once quickly followed by 3× for 15 min in 1× PBS.
8. Photograph embryos (*see* **Note 13**).
9. Optional: Embryos may be cryoembedded in gelatin (recommended) or OCT (Subheadings 3.1.3 and 3.1.2, respectively) and sectioned following detection in whole mount.

3.4.2 ß-Galactosidase Detection in Sections

1. Prepare OCT sections as described in Subheading 3.1.2.
2. Thaw sections and wash in PBS for 5 min.
3. Post-fix for 10 min in freshly made 4% paraformaldehyde at 4 °C.
4. Wash slides in ß-gal washing buffer once quickly, and then 2× for 10 min.
5. Incubate slides in ß-gal detection buffer at 37 °C, protected from light, until color develops (1 h - overnight). Reaction should be closely monitored for the first several hours to determine its rate.

6. Wash slides in PBS quickly, and then 3× for 10 min, or until solution stops turning yellow.
7. Mount slides in gelvatol as described in Subheading 3.3.2, **step 14**, and photograph.

3.4.3 Alkaline Phosphatase Detection in Whole Mount

1. Dissect embryos or tissues into ice-cold PBS. For stages after the neural tube has closed, puncture the neural tube to prevent trapping of reagents. Transfer to glass vials (*see* **Note 6**).
2. Incubate embryos from 2 h to overnight in 4% paraformaldehyde, rocking.
3. Wash embryos in PBT, 2× quickly followed by 3× 10 min, rocking.
4. Incubate embryos at 65 °C for 1-several hours, in PBT, to inactivate endogenous phosphatases. Embryos do not need to be rocking during this step. The length of incubation depends on how highly expressed AP is (lower expression will require more inactivation of background-producing phosphatases) (*see* **Note 30**).
5. Wash embryos in NTM, 1× quickly and 1× 10 min, rocking.
6. Incubate embryos in detection buffer (NTM containing NBT and BCIP), rocking at room temperature, in the dark, until signal has developed. Monitor the reaction closely to determine speed, which can take between a few minutes and many hours. If reaction proceeds very slowly, it can be left at 4 °C overnight. Staining is complete when the staining of interest is clearly visible, or when background begins to come up at the same rate at which the signal is developing. For whole mount, it is unlikely that improvement will be seen for embryos incubated longer than overnight at 4 °C.
7. Wash embryos in PBT 1× quickly followed by 3× 10 min, rocking.
8. Post-fix embryos in 4% paraformaldehyde for 30 min, rocking.
9. Wash embryos in PBT 1× quickly followed by 3× 10 min, rocking.
10. Optional: If background has developed too much, embryos can be washed overnight at 4 °C in PBS containing 1% Tween-20.
11. Photograph embryos (*see* **Note 13**).

3.4.4 Alkaline Phosphatase Detection in Sections

1. Prepare either paraffin or cryosections, as described in Subheadings 3.1.1–3.1.3.

   Steps 2–6 are only necessary for paraffin sections.
2. Bake slides on hot plate at 60 °C for 1 h.
3. Allow slides to come to room temperature.

4. Dewax in xylene 2× for 5 min.
5. Wash 2× for 5 min in 100% methanol.
6. Rehydrate sections through a graded ethanol series with 5-min washes at room temperature, as follows:

   100% Methanol.

   100% Methanol.

   75% Methanol/dH_2O.

   50% Methanol/PBS.

   25% methanol/PBS.

   PBS

   PBS

   For cryosections, thaw slides and air-dry for 15–20 min at room temperature.
7. Heat-inactivate endogenous phosphatases by incubating slides in PBT at 65 °C for 30 min.
8. Incubate slides in NTM buffer for 10 min at room temperature.
9. Using a pap pen, draw a line across the right edge of the slide label, to prevent fluid from covering the label. Blot slides by touching the bottom edge to a paper towel and lay them flat inside a humidified staining tray (*see* **Note 5**).
10. Gently pipette 1 mL of detection buffer onto each slide, taking care to avoid pipetting directly onto tissue. Cover humidified tray and incubate until signal has developed (several minutes-hours or, very rarely for a transgene, 2–3 days).
11. Wash slides in PBT once quickly, and then three times for 5 min.
12. Post-fix in 4% paraformaldehyde for 30 min-overnight.
13. Wash slides in PBT once quickly, and then three times for 5 min.
14. Mount slides in gelvatol and photograph.

3.4.5 Direct Detection of Fluorescent Reporters in Whole Mount

1. Dissect embryos or tissues into ice-cold PBS (*see* **Note 31**).
2. If possible, observe and photograph embryos immediately, as the signal is strongest in fresh tissue (*see* **Note 32** for GFP filter sets).
3. 3. Optional: Transfer to glass vials (*see* **Note 6**) and fix embryos in 4% paraformaldehyde for 30 min to 2 h at 4 °C. It is important to keep the fix as short as possible to preserve GFP fluorescence (*see* **Notes 31** and **33**).

4. Wash embryos in PBS 1× quickly, and then 3× for 10 min in PBS.
5. Photograph embryos (*see* **Note 13**).

3.4.6 Detecting Fluorescent Reporters in Sections

1. Prepare OCT sections as described in Subheaing 2.2.2.

   Optional: For better morphology, a method for directly visualizing GFP in paraffin sections has been reported [2].
2. Thaw slides and wash briefly in PBS.

   Optional: Detect FP via IHC, using Subheading 3.3.2. If combining FP detection with IHC that requires organic solvents or antigen retrieval, this will denature FPs and require use of IHC.
3. Mount in anti-fade mountant or gelvatol as described in Subheadings 3.3.2, **step 14**.

4 Notes

1. Hazardous Chemicals

   Acetic anhydride is flammable and harmful if inhaled. It can cause burns to the respiratory tract, skin, and eyes upon contact. Wear suitable protective clothing/equipment and work with acetic anhydride in a fume hood.

   Benzyl alcohol/benzyl benzoate (BABB) is harmful if swallowed, and is an irritant to the skin, respiratory tract, and eyes.

   CHAPS is an irritant to the skin, eyes, respiratory tract, and mucosal membranes. Strongly avoid breathing the dust. Wear appropriate protective clothing/equipment.

   Chloroform is highly volatile and is an irritant and carcinogen. Wear suitable protective clothing/equipment and work with chloroform in a fume hood.

   Diethyl pyrocarbonate (DEPC) produces toxic, corrosive, flammable, or explosive gases. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment and only work with DEPC in a fume hood.

   Diaminobenzidine (DAB) is a carcinogen. Wear suitable protective clothing/equipment and handle in a fume hood.

   Formamide is toxic and is harmful if inhaled. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment. Formamide-based solutions, especially those heated to 65 °C, should be handled in a fume hood.

Glutaraldehyde is toxic. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment and work with glutaraldehyde in a fume hood.

Methanol is toxic and flammable. It is an irritant to the skin and eyes and is harmful to many organ systems if ingested. Wear suitable protective clothing/equipment and handle only with sufficient ventilation.

2-Methyl butanol is flammable and an irritant. Avoid breathing vapors, and wear appropriate protective clothing/equipment.

Paraformaldehyde is toxic; strongly avoid breathing the dust. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment and work with paraformaldehyde in a fume hood.

Phenol is toxic and will cause burns if exposed to skin or eyes. Wear suitable protective clothing/equipment and work with phenol in a fume hood.

Sodium dodecyl sulfate (SDS) is an irritant to the skin, eyes, respiratory tract, and mucosal membranes. Strongly avoid breathing the dust. Wear appropriate protective clothing/equipment.

Triethanolamine (TEA) is an irritant to the skin and eyes, and can cause liver and kidney damage. Wear appropriate protective clothing/equipment and work with TEA in a fume hood.

Xylene is flammable and toxic and should be handled only in a fume hood.

2. To TESPA treat: take clean, plain glass slides through the following washes, in glass staining dishes: 1 min in 2 N HCl, 1 min in H_2O, 1 min in acetone, <30 s acetone/2% TESPA (2-aminopropyltriethoxysilane, Sigma), 30 s in acetone, 30 s in ethanol, then air-dry overnight at room temperature, protected from dust. 2% TESPA solution should be made fresh before use. Use dedicated glass slide dishes and glass pipettes for treatment, as all will become TESPA coated.
3. Considerations for designing riboprobes: In general, probes of 400–700 bp are designed though they are certainly not restricted to this size. Probes are often designed to encompass the 3'UTR of a gene to limit cross-reactivity of the probe with homologous genes. Potential alternate splicing of the target sequence should be considered.
4. Single-, double- and triple-in situ hybridization reactions can be performed simultaneously to detect up to three unique RNA transcripts. Each RNA riboprobe must be differentially

labeled with either digoxigenin (DIG)-, fluorescein (FL)-, or biotin-conjugated UTP and all probes are added simultaneously to the hybridization reaction. Detection of each uniquely labeled riboprobe must be performed sequentially, with a short 4% paraformaldehyde fixation step in between each to inactivate previous AP activity. A common method for triple detections is as follows:

(a) Detect DIG using AP-conjugated anti-DIG antibody. Anti-DIG-AP then catalyzes the conversion of NBT/BCIP to a purple precipitate.

(b) Detect FL using AP-conjugated anti-FL antibody. Anti-FL-AP then catalyzes the conversion of INT/BCIP to a brown precipitate.

(c) Detect biotin using AP-conjugated anti-biotin antibody. Anti-biotin-AP then catalyzes the conversion of Sigma Fast Red TR/Naphtali AS-MX to a pink precipitate.
As FL and biotin-labeled riboprobes exhibit somewhat reduced signal, it is best to generate these riboprobes against genes of interest that exhibit the most robust signal.

5. For section procedures, steps using limiting/expensive reagents can be carried out on horizontal slide staining trays at lower volume. Trays can be purchased or made by fixing rails (plastic stripettes) to the bottom of a flat, covered dish. We use 240 × 240 × 20 mm QTrays (Genetix). Wet paper towels or Whatman paper is placed in the bottom of the tray to keep it humidified. 1 mL of liquid per slide is sufficient in a covered, humidified tray overnight. 100 μL can be used if slides are covered with a plastic coverslip (cut from thick polyethylene bags (Bel Art)). These should be floated off gently in buffer, rather than pulled off, to minimize tissue damage.

6. Unless otherwise noted, incubations on whole-mount embryos and tissues should be done in 5–10 volumes (or more) of solution greater than the volume occupied by the tissue, on a rocking platform. We use 4 or 10 mL screw-top scintillation vials (VWR). For slides, we use 5-slide mailers (approximately 20 mL) or 25-slide buckets (approximately 200 mL) (VWR).

7. To fix embryos into positions (such as with a straight body axis), which can make sectioning easier, fixation can be carried out in Petri dishes with black dissection wax in the bottom, and embryos can be pinned out with insect pins. Embryos fixed this way will hold their position during subsequent processing.

8. To prevent degradation of RNA riboprobe, all steps up to and including hybridization should be performed under RNase-free (RF) conditions. Consumables such as Eppendorf tubes

and pipette tips can be purchased RNase free. Stock solutions such as dH_2O and 10× PBS should be treated with 0.1% diethyl pyrocarbonate (DEPC), which covalently modifies and inactivates RNase. DEPC is highly toxic and should only be added in a fume hood. Once added, stir vigorously overnight. DEPC must be inactivated by autoclaving prior to use.

9. Following riboprobe synthesis, a RNA band of approximately tenfold greater intensity than the plasmid band indicates that approximately 10 mg of probe has been synthesized. There may be more than one band due to secondary structure but there should not be a smear. A smear may indicate probe degradation (if smear is below probe band) or synthesis from uncut plasmid (if smear is above probe band).
10. Efficient hybridization requires overnight incubation at 65 °C with constant gentle agitation. This is most easily achieved using screw-top scintillation vials (VWR or Wheaton; 4 mL or 20 mL depending on the embryo size and number) placed in a heated orbital shaking water bath. If this is not available, embryos can be placed in a 2 mL round-bottom RNase-free Eppendorf tube, parafilm sealed, and placed in tube holder (e.g., water bath tube holder). This holder is placed inside a hybaid bottle such that it is fixed in place, and incubation performed in a hybridization oven with constant rotation.
11. In general, a hybridization temperature of 65 °C yields strong signal with minimal background. If no signal is observed, the hybridization temperature should be lowered, initially to 60 °C and then 55 °C if necessary. Conversely, if high background is observed, the hybridization temperature can be increased to 70 °C.
12. In the past, preadsorption of anti-DIG antibody with embryo powder was performed to remove nonspecific antibodies. This is no longer routinely performed but may be helpful if high background levels are observed.

    Generation of embryo powder

    (a) Homogenize ~12.5–14.5 dpc mouse embryos in a minimum volume of PBS.

    (b) Add 4 volumes of ice-cold acetone and mix.

    (c) Incubate on ice for 30 min.

    (d) Centrifuge at 8,000 × *g* for 10 min and remove supernatant.

    (e) Wash pellet with ice-cold acetone (taking care to resuspend the pellet well) and spin again.

    (f) Spread the pellet out and grind into a fine powder on a sheet of filter paper. Allow to air-dry.

    (g) Store in an airtight tube at 4 °C.

Preadsorption of anti-DIG antibody with embryo powder

(a) For each 2 mL required, weigh out 1.5 mg of embryo powder and add 0.5 mL of antibody blocking solution and 1 μL of anti-DIG antibody.

(b) Rock gently at 4 °C for 2 h or longer.

(c) Centrifuge at maximum speed for 10 min at 4 °C.

(d) Dilute the supernatant to 2 mL using antibody blocking solution.

(e) Store at 4 °C until required.

13. Whole-mount embryos can be photographed on a standard dissecting microscope, either in depression chambers or in Petri dishes and submerged in buffer (such as PBS). Placing 20–30 mL solidified 2% agarose/PBS in the bottom of the dish allows the embryo to be positioned, either by propping them in holes made in the agarose, on top of chunks of agarose, or by immobilizing with insect pins.

14. Enzymatic detection (HRP or AP) gives higher signal than does fluorescence, and is generally easier to optimize for whole mounts. If background from endogenous enzymes is an issue, it can be reduced somewhat by extensive washing in PBT or PBS + 1% Tween-20 or Triton X-100 (overnight or longer) after detection, and can also be reduced by clearing embryos in BABB or glycerol.

15. An overnight fixation step is usually acceptable for whole-mount IHC; however, overfixation can increase epitope masking, and aldehyde fixation, especially glutaraldehyde, and also autofluorescence. It is therefore sometimes useful to shorten fixation to a few hours at 4 °C. Alternatively, the weaker Dent's fix (1 part DMSO to 4 parts methanol) can be used in place of aldehydes by incubating for 24 h.

16. Dent's bleach can be replaced with Dent's fix (1 part DMSO to 4 parts methanol) if not using HRP detection, where it is necessary to quench endogenous peroxidases.

17. Weak proteinase digestion is an alternative to permeabilization in Dent's and can be used in place of **steps 4–5** in the protocol above. For HRP detection, tissue must still be bleached in 3% H_2O_2 in methanol prior to rehydration. Next, incubate embryos in 10 μg/mL proteinase K (Roche) in PBS. Make fresh from a stock solution of 10 mg/mL proteinase K, stored at −20 °C. Incubate embryos for 5–10 min (time must be optimized for each antibody to balance permeabilization with antigen degradation). Stop digestion with two rapid washes in 2 mg/mL glycine in PBS. Make glycine solution fresh on the day of use. Proceed to **step 7** (Subheading 3.3.1).

18. The following modifications are used for enzymatic detection with an AP-conjugated secondary antibody:
    (a) After **step 7**, denature endogenous phosphatases by heating sections to 65 °C for 1-several hours. Note that this will also destroy some antigens.
    (b) After **step 12**, incubate slides in NTM buffer for 2× for 5 min.
    (c) Lay slides flat in a humidified staining tray (*see* **Note 5**). Gently pipette 1 mL of NTMT including 3.5 μL of 100 mg/mL NBT (Roche) and 3.5 μL 50 mg/mL BCIP (Roche) per mL. Alternatively, use of the premixed substrate solution BM Purple (Roche) is highly recommended as background staining is significantly reduced.
    (d) Cover humidified tray and incubate until signal has developed (several minutes-hours or, very rarely for a transgene, 2–3 days). 4 °C incubation will inhibit background color.
    (e) Wash with NTM (5–10 min, do not leave too long) and then PBTX for 20 min.
19. Secondary-only controls (omitting only the primary antibody) should be performed for all IHC procedures.
20. The precipitate formed by DAB is brown, and can be made a more visible dark gray by adding metal salts to the detection solution [1].
21. BABB dissolves plastic so glass dishes and tubes must be used. Additionally, BABB dissolves the precipitate formed by AP detection, so if it is used photography must be done immediately. Glycerol is a weaker but nontoxic clearing agent, and is easier to use with AP detection. Incubate embryos in 50% glycerol/PBS, rocking at room temperature, until equilibrated (until they sink); then replace with 100% glycerol; and equilibrate again.
22. Section IHC in OCT-embedded tissues gives the best signal and requires less time to embed and stain. Though morphology is better in paraffin, signal tends to be lower, and more invasive permeabilization techniques are needed. Some antibodies perform best when OCT-embedded without fixation. This "fresh-frozen" method is not compatible with detection of reporters, which require fixation. Finally, if the heat or organic solvents required for embedding destroy an antigen then tissues can be stained in whole mount, and subsequently embedded.
23. The following modifications are used for enzymatic detection with HRP/DAB:

(a) After **step 2**: Quench endogenous peroxidases by incubating for 1 h in 3% H_2O_2 in methanol.

(b) After **step 10**: Lay slides flat in a humidified staining tray (*see* **Note 5**). Gently pipette 1 mL slides in HRP detection buffer (1:1 mixture of 0.04% H_2O_2 in water and 1 mg/mL diaminobenzidine in 100 mM Tris–HCl, pH 7.2; *see* Subheading 2) and observe closely to determine the rate of color development. This can begin within seconds, but can also take minutes to several hours.

(c) Wash with PBT at least 3× for 10 min to stop reaction.

24. Permeabilization note: While many antibodies function without permeabilization, most require this step for strong signal (antibodies against extracellular antigens, for example, can often access targets without help). Antigen retrieval, Triton-X permeabilization, and enzymatic digestion are commonly used techniques to unmask epitopes. Antigen retrieval (AR) involves heating of the tissue in a citrate-based buffer, which reverses protein cross-links formed during fixation. This method is commonly used for nuclear and intracellular antigens. Boiling damages tissue, but can be avoided with a pressure cooker and, with proper precautions, morphology can be preserved. If AR is required, cut thin sections (<12 μm), subsequently dry at 37 °C, and after performing AR allow the pressure cooker to cool before removing the lid. Finally, AR denatures fluorescent reporters, and so IHC must be used to detect them.

    Although effective, AR is relatively time consuming and technically more challenging than permeabilization with Triton-X, a powerful detergent that dissolves membranes, allowing antibodies to enter both cells and organelles. Many antibodies produce beautiful signal when Triton-X is added to blocking, primary, and secondary antibody solutions (Fig. 1b). However, some are sensitive to detergents, and will not work if present. Antibodies against transcription factors/nuclear antigens that must traverse cellular and nuclear membranes often respond best to Triton-X, but this is not always the case and must be determined empirically. Enzymatic digestion is a third option for revealing epitopes. Proteinase K is commonly used, but trypsin and pepsin are alternatives. If an antibody is sensitive to Triton-X, or a reporter protein must be preserved, enzymatic retrieval is a sound choice. Note, however, that prolonged incubation, high concentration of enzyme, or failure to inactivate digestion can disturb tissue morphology and resulting signal. *See* [10] for a full discussion of antigen retrieval techniques, and their advantages and drawbacks.

25. To expose epitopes by antigen retrieval: Fill pressure cooker with citrate buffer, lock lid, and add red weight; microwave pressure cooker to preheat (approximately 15 min on high in our microwave). Place slides in a vertical slide staining rack inside the pressure cooker, taking care to fully submerge them. Fasten lid and microwave pressure cooker on high. A hissing sound indicates when sufficient pressure is reached (in our microwave, ~5 min). Continue to microwave for 10 min after hissing becomes audible. Incubate locked pressure cooker at room temperature until pressure indicator (yellow stem) falls (approximately 15 min). Do not open when pressurized, as this can damage the tissue. Open pressure cooker, ensure that slides are still submerged, and allow them to cool for 40 min. Wash 2× for 5 min in PBS.
26. To expose epitopes by Triton-X permeabilization, prepare 0.1–0.5% Triton-X/PBS, rocking solution for 10 min to mix. Incubate slides in solution, 10–30 min.
27. To expose epitopes by enzymatic digestion, prepare 1–10 μg/mL proteinase K in PBS (concentration determined empirically), diluted fresh from a 10 mg/mL stock, stored at −20 °C. *See* **Note 24** for a list of substitute enzymes. Incubate slides for 2–5 min (time determined empirically). Wash slides once quickly and then 2× for 5 min in 2 mg/mL glycine/PBT (prepared fresh) to stop the digestion.
28. Mouse-on-mouse immunohistochemistry (MOM) is the staining of mouse tissue with primary antibody raised in mouse. MOM is prone to background because the secondary recognizes immunoglobulins (IgGs) endogenous to the tissue. To reduce background, preincubate tissue with anti-mouse IgG fab fragments during the block step. Whole IgGs should be avoided, as the second site can trap and thus decrease available primary antibody in the tissue.
29. Although it is stable in vivo, ß-galactosidase (ß-gal) is sensitive to aldehyde fixation, organic solvents, and heat. Key considerations are minimizing fixation time, keeping tissues cold, and processing them as quickly as possible. When ß-gal is to be detected along with other markers (such as an AP reporter or IHC using any detection method), the ß-gal detection should be done first to minimize loss of signal. Staining in whole mount followed by gelatin embedding and sectioning is recommended, but it may not be possible to develop signal deep inside larger embryos/tissues. OCT embedding is the best method if tissues are to be stained in section because it does not require organic solvents or heat, and is least damaging to the signal. Paraffin-embedded sections cannot be stained for ß-galactosidase.

30. Human placental alkaline phosphatase is a robust and stable reporter of gene expression that can be readily detected in whole-mount or embedded and sectioned tissues. Alkaline phosphatase retains its activity following aldehyde fixation (even for long periods), in organic solvents including methanol and ethanol, and after extended heating. The primary consideration for detecting alkaline phosphatase reporters is inactivating endogenous phosphatases in tissues, which can lead to high background. This is accomplished by heating tissues to 65 °C, a temperature at which mouse but not human placental alkaline phosphatase is inactivated.
31. Preserving and detecting exogenous fluorophore signal while minimizing autofluorescence is the key consideration for visualizing FP reporters. Many embryonic tissues emit autofluorescence, especially blood, lung, liver, and other gut tissues, and this is particularly true in the green and red channels. Autofluorescent blood cells are easily identifiable as large, round cells dispersed randomly throughout the tissues. Aldehyde fixation (especially glutaraldehyde) increases background fluorescence and also weakens FP signal, so fixation, if it is required at all, should be kept to a minimum. As an alternative to direct visualization, commercial antibodies against FPs are available, and can be used with standard IHC techniques. We recommend anti-RFP (Rockland Immunochemicals, 600-401-379), anti-GFP (abcam, ab290) which also recognizes YFP, and anti-tRFP/TagBFP (Evrogen, AB233). For multi-FP reporter detection, antibodies tested for lack of cross-reactivity are available (Kerafast, EMU109). New FPs are continually being developed (see for example http://www.fpvis.org/FP.html).
32. Enhanced GFP (EGFP) can be detected with a standard FITC filter set. If background fluorescence is a problem, a long-pass filter set specialized for EGFP excitation can be used instead (Nikon or Chroma Technologies). With this filter, background autofluorescence appears yellowish-green while EGFP is very bright green. The suppliers above also make a variety of GFP filter sets optimized for different color variants and combinations.
33. There have been some reports in cultured cells that the subcellular pattern of GFP fluorescence can change following fixation [16]. If this is an important consideration, then GFP distribution should be examined in fresh compared with fixed tissues.

Acknowledgments

We are deeply grateful to Connie Cepko, Cliff Tabin, Carol Wicking, and to past and present members of the Cepko and Tabin labs, who developed most of the protocols as they are described here, and who have generously shared them with us. We thank Lucie Jeannotte and Kim Landry-Truchon for advice and improvements to immunohistochemistry methods. The Australian Regenerative Medicine Institute is supported by grants from the State Government of Victoria and the Australian Government. This work was supported by NH&MRC grants APP1051792 and APP1063967 to E.M.

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Chapter 13

Reptile Embryology and Regeneration

Matthew K. Vickaryous and Emily A. B. Gilbert

Abstract

Reptiles (lizards, snakes, turtles, and crocodilians) are becoming increasingly popular as models for developmental investigations. In this review the leopard gecko, *Eublepharis macularius*, is presented as a reptilian model for embryonic and tissue regeneration studies. We provide details of husbandry and breeding and discuss aspects of embryonic nutrition, egg anatomy, and sex determination. We provide comprehensive protocols for transcardial perfusion, short-term anesthesia using the injectable anesthetic Alfaxan, and full-thickness cutaneous biopsy punches, used in geckos for the study of scar-free wound healing. We also provide modifications to three popular histological techniques (whole-mount histochemistry, immunohistochemistry, and double-label immunofluorescence) and provide details on bromodeoxyuridine (BrdU) labeling and immuno-detection.

Key words Reptilia, Gecko, Husbandry, Embryo, Sex determination, Anesthesia, Biopsy, Perfusion whole-mount histochemistry, Immunohistochemistry, Immunofluorescence, Proliferating cell nuclear antigen, Vimentin, Bromodeoxyuridine

1 An Introduction to Reptiles

Reptiles are a diverse radiation of amniotes with more than 10,450 recognized species [1]. In the modern sense Reptilia includes all amniotes that are not mammals (or more accurately "the most inclusive clade containing *Lacerta agilis* Linnaeus 1758 and *Crocodylus niloticus* Laurenti 1768, but not *Homo sapiens* Linnaeus 1758") [2]. Consequently, birds are reptiles. Historically however, reptiles (as a group) have excluded both birds and mammals. To avoid unnecessary confusion, this contribution will focus on reptiles in the classical or structural sense: a group distinguished by keratinized scales and poikilothermy.

Modern reptiles include turtles and tortoises (Testudines), alligators, crocodiles and relatives (Crocodylia), tuatara, snakes, and lizards (Lepidosauria). Testudines are easily recognized by the unique presence of a carapace, plastron ("shell") and a pectoral apparatus deep to the ribcage. Crocodilians are large, semiaquatic predators with robust tails and thecodont dentition (teeth set in

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_13,

sockets). Lepidosauria are enormously diverse and include limbed forms, numerous unrelated species with varying degrees of limb reduction, as well as independently evolved limbless groups. Snakes (Serpentes) are the best known group of limbless lepidosaurs. Other limbless groups include many species of lizards and amphisbaenians, a group of elongate burrowing forms with robust skulls and annulated skin (giving members an earthworm-like appearance). Although superficially lizard-like, tuatara represent the outgroup to other lepidosaurs and demonstrates various primitive features including an akinetic skull (with a well-developed lower temporal bar) and the absence of hemipenes. Lepidosaurs exclusive of tuatara are known as squamates (Squamata). The term "lizard" is typically used to denote a non-serpent, non-amphisbaenian squamate.

Until recently, species selection was often limited to locally available taxa. However, increasing numbers of reptiles are becoming widely obtainable due to their popularity in the pet trade, including Eastern corn snakes (*Pantherophis guttatus*), bearded dragons (*Pogona vitticeps*), anoles (*Anolis* spp.), and various geckos (*Eublepharis macularius*, *Paroedura pictus*). In addition, breeders of exotic species may provide unique opportunities to investigate less common reptiles (e.g., African rock pythons, *Python sebae*) [3, 4]. For many reptiles, especially large, semiaquatic species (e.g., crocodilians, various turtles), it is more practical to collect eggs from wild populations or commercial farms and then artificially incubate them in a controlled environment. If considering captive husbandry it is essential to investigate the species-specific environmental requirements (*see* **Note 1**).

Most reptiles, including all turtles, crocodilians, tuatara, and majority of squamates, produce cleidoic eggs following internal fertilization, a pattern of reproduction known as oviparity [5]. As the greater part of development occurs outside the body, oviparous development is particularly sensitive to changes in environmental conditions, specifically temperature [6]. Consequently, the incubation period may vary considerably both between and within species [7]. Furthermore, many reptiles demonstrate temperature-dependent sex determination (*see* **Note 2**). Oviparous species are lecithotrophic, obtaining the majority of their nutrients from the yolk mass [5]. Eggs produced by oviparous species are enclosed by a series of membranes and a calcareous shell that varies in structure (*see* **Note 3**). Depending on the species, the eggshell or the yolk sac may act as the primary source of calcium.

As an alternative to oviparity, various squamates have independently evolved viviparity: in utero embryonic development [7]. More specifically, viviparity refers to embryonic development that combines the nutritional features of both placentotrophy, in which the placenta provides nutrients to the developing offspring, and lecithotrophy, in which the yolk sac is responsible for providing

nutrients to the developing offspring [8]. The term ovoviviparity was traditionally used to describe a mode of reproduction wherein the embryos obtained a majority of their nutrients from the yolk sac, but used the placenta for gas exchange [9]. More recently however, ovoviviparity has been recollected as part of the viviparous spectrum of development. The proportion of nutritional elements that are obtained from the yolk sac and placenta varies among species. For instance, scincid lizards, members of the genera *Pseudemoia* and *Niveoscincus,* rely more heavily on placentotrophy than lecithotrophy to obtain nutrients [5]. The species *Eulamprus tympanum,* on the other hand, rely more heavily on lecithotrophy than placentotrophy [10].

Related to the increasing interest in reptilian development, embryonic staging tables are now available for various turtles [11–13], crocodilians [14, 15], snakes [16, 17], and lizards [18–20]. Although embryos of reptiles such as tuatara and amphisbaenians remain difficult to acquire, staging tables have been published [21, 22]. This chapter describes husbandry procedures for keeping and breeding leopard geckos, *Eublepharis macularius*, a tractable, laboratory-appropriate oviparous lizard. In addition we provide details on two common techniques for use with reptile embryos: whole-mount histochemistry and serial immunohistochemistry.

1.1 Husbandry and Embryogenesis in Leopard Geckos

Leopard geckos (*Eublepharis macularius*; Fig. 1a) are one of the most popular reptiles in the pet trade. Adults are an average of 120 mm in body length with a docile temperament and have minimal husbandry requirements. Males are typically more robust than females and demonstrate a conspicuous "V"-shaped row of femoral pores (immediately cranial to the vent) and hemipenile swellings. Clutch size is small (most commonly two), but females can have multiple clutches throughout a season [20]. Leopard geckos use temperature-dependent sex determination, and the temperatures for producing males and females are documented [23]. Furthermore, an embryonic staging table of in ovo development has recently been published [20].

Female leopard geckos may be kept singly or in small groups [2–10] depending on the enclosure size. Males should be kept singly or with one or more females for the purposes of breeding. Males may fight if housed together. A standard rat-sized nalgene or polycarbonate enclosure (~23 cm × 42 cm × 19 cm high), with a lid (either perforated polyvinyl chloride (PVC) sheeting or stainless steel bars), water bowl, two hide boxes, and a layer of newspaper is suitable for 1–2 adult individuals (Fig. 1b). A weigh boat with powdered supplement should be provided to sexually immature (and rapidly growing) subadults and gravid adult females. Although lacking subdigital adhesive pads, leopard geckos are capable climbers and can squeeze through relatively small openings. Leopard

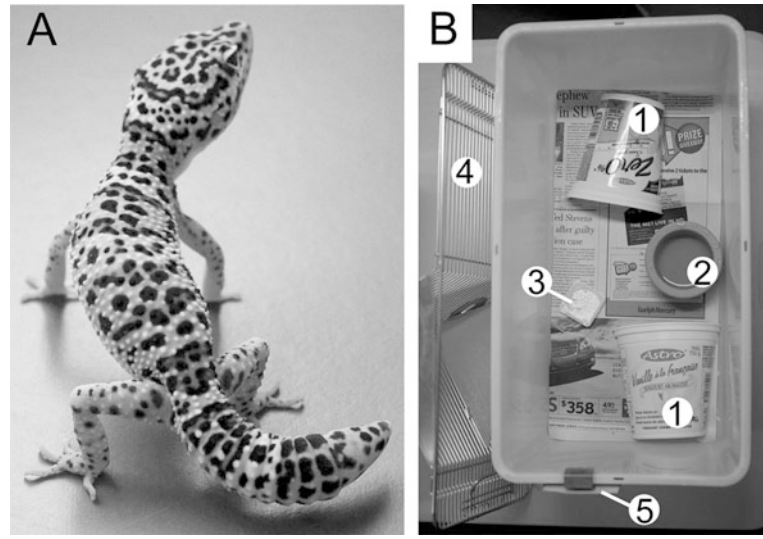

Fig. 1 (**a**) Adult female leopard gecko, *Eublepharis macularius*. As for all eublepharids (but unlike most geckos) leopard geckos have movable eyelids and lack adhesive toe pads. (**b**) Leopard gecko laboratory enclosure. One or two adult leopard geckos can be housed in a standard rat-sized cage lined with newspaper or paper towel. Each enclosure should include (1) two hide boxes, (2) water bowel, (3) a dish of powdered supplement (calcium and vitamin D_3), and (4) a lid (in this case, a stainless steel mouse-gauge wire top). (5) Card holders and cage labels are useful for organizing larger colonies

geckos are adept at escaping so we recommend using perforated PVC sheeting, sized to cover the enclosure. If using mouse-gauge wire lids (distance between bars = 1 cm), then the hide boxes should be short so to prevent individuals from reaching the wire lid. Avoid using wire lids with a built-in cradle for food and water bottle or invert these lids. Two hide boxes should be provided, one at either end of the enclosure. As for many reptiles, leopard geckos seem to prefer enclosed spaces so hide boxes need only be large enough for the number of individuals present. Individuals are fed a diet of gut-loaded mealworms or crickets, both of which should be dusted with powdered supplement every 1–3 days. Subadult geckos will eat three to five regular-sized mealworms (ranging between 1 and 10 mealworms) daily. Sexually mature adults can be fed larger numbers of mealworms less frequently. Although most individuals establish a latrine site, enclosures should be replaced weekly and given a change of newspaper, a clean water dish, and new hide boxes. Ambient temperature can range between 22 and 32 °C with a preferred body temperature of 25.8 °C [24]. The establishment of a heat gradient is important for behavioral thermoregulation. The heat cable can be placed under one end of the enclosure (below one of the hide boxes). Our colony is organized using a rack

system with multiple enclosures (Fig. 1c) and kept at an ambient temperature of 24 °C (temperatures over the heat cable averaging 3–4 °C above ambient), on a light cycle of 12:12 with an ambient humidity of 40–50%. Hatchling geckos often have difficulty removing shed skin from toes. If necessary, soak the feet in room-temperature water to hydrate shed skin and carefully remove with forceps. With regular handling leopard geckos become tame and docile.

Females have a preference for nest sites between 28 and 29 °C [25]. One of the hide boxes can be converted into a nesting chamber by the addition of 1:1 vermiculite (or other water-absorbing material) and water to provide an area of elevated humidity. Once eggs are deposited they should be collected and incubated in a commercial avian incubator. Leopard geckos use temperature-dependent sex determination (TSD). Females are produced at low temperatures (26–28 °C), mostly females (75%) at high temperatures (34 °C), and mostly males (~89%) at intermediate temperatures (31.5 °C) [23]. Between these temperatures both males and females are produced. Humidity during the incubation period should be maintained at close to 80%.

Embryogenesis (Fig. 2) is well under way prior to laying (oviposition) and, similar to avians, absolute time offers only a rough guide to state of development. As noted previously, rate of development is strongly influenced by incubation temperature. The role of other factors, including passage time within the oviduct, egg retention, and humidity of the incubator, may also influence the developmental stage at oviposition. Although details remain unclear, gastrulation and neurulation are predicted to be

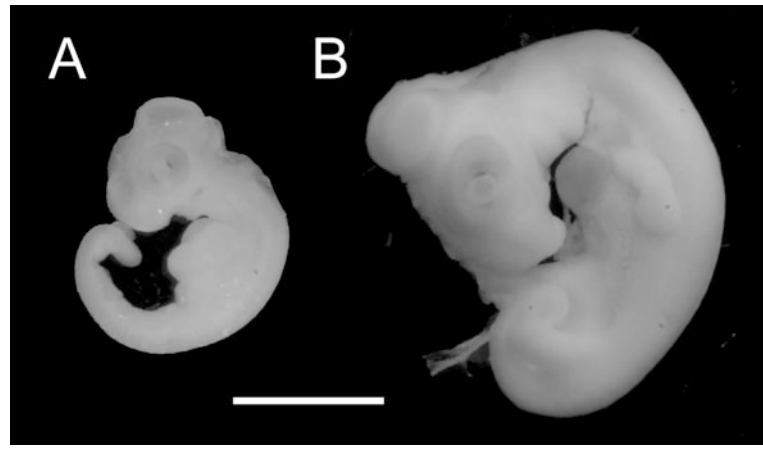

Fig. 2 Leopard gecko embryos. (**a**) Stage 29, at or shortly after oviposition. At this stage both forelimb and hindlimb buds are present. (**b**) Stage 31. The forelimb buds have developed a paddle-shaped autopodium. *See* [19] for details. Scale bar = 2 mm

comparable with the events observed in avians. In leopard geckos, oviposition typically occurs at stage 28, characterized in part by the presence of forelimb buds (but not hindlimb buds) and incomplete closure of the choroid fissure of the developing eye. This correlates with avian embryos at Hamburger and Hamilton (HH) stage 17 [20]. At a constant temperature of 28 °C ± 1 °C, the duration of in ovo development for leopard geckos is an average of 52 days.

1.2 Transcardial Perfusion for Fixation of Subadult and Adult Leopard Geckos

Transcardial perfusion allows for efficient and consistent fixation of whole subadult and adult geckos [26]. This process removes all blood products (exsanguination) and is followed by the injection of fixative throughout the entire animal. Transcardial perfusion is required for tissue preparation prior to immunohistochemistry and immunofluorescence. Prior to perfusion, animals are chemically euthanized using ethyl 3-aminobenzoate methanesulfonate salt (tricaine methanesulfonate; MS222). MS222 is commonly used as an anesthetic and a chemical euthanasia method for fish, amphibians, and lizards [27]. MS222 blocks the generation of action potentials by modifying the properties of voltage-gated Na^+ channels, thus depressing both respiration and cardiac function [28].

1.3 Anesthesia for Postnatal Leopard Geckos Using Alfaxan (Alfaxalone)

Alfaxan is a synthetic neuroactive steroid that has been successfully used in clinics to achieve a surgical anesthetic plane in various reptiles including tortoises (*Agrionemys horsfieldii*; [29]), green iguanas (*Iguana iguana*; [30]), and leopard geckos (*Eublepharis macularius*; [31]). Alfaxan induces anesthesia through activity on $GABA_A$ receptors in the central nervous system. It works by overpolarizing neurons and inhibiting action potential propagation. We have determined that the optimal dose of Alfaxan is 30 mg/kg in a divided dose delivered bilaterally on either side of the rostral spinal cord into the epaxial muscles. Average induction time for leopard geckos is 3 min with a plateau of 12–15 min and a recovery of 20 min in an environmental chamber with an ambient temperature of 28 °C ± 1 °C.

1.4 Full-Thickness Cutaneous Biopsy Punch for Postnatal Leopard Geckos

Full-thickness cutaneous biopsy punches allow for the study of wound healing in leopard geckos. We have successfully used 3 mm wounds on both the dorsal surface of the body and tail. Prior to biopsy, animals are anesthetized (*see* Subheading 1.3) and the location of the biopsy is determined and marked using a nontoxic permanent marker. Following anesthetic induction, the biopsy is performed using a slow twist of the biopsy tool (Fig. 3a, b), followed by the removal of the punch with forceps (Fig. 3c) and a scalpel blade to reveal the open wound (Fig. 3d).

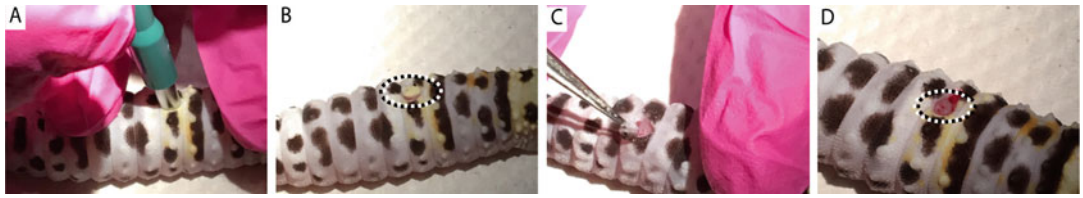

Fig. 3 Biopsy punch on the dorsal tail of the leopard gecko, *Eublepharis macularius*. (**a**) The biopsy tool is placed perpendicular to the chosen biopsy location. (**b**) Slowly twisting the biopsy tool creates a puncture around the entire circumference of the epidermis and dermis of the 3 mm biopsy punch. (**c**) Forceps are used to remove the tissue plug. (**d**) The open 3 mm biopsy wound is exposed

1.5 Whole-Mount Histochemistry: Single (Alizarin red) and Double (Alizarin red and Alcian blue) Embryo Staining Protocol

Whole-mount clearing and staining commonly involve double staining with Alizarin red S for calcified structures in combination with Alcian blue 8GX for tissues rich in glycosaminoglycans [32]. However, the Alcian blue solution uses glacial acetic acid as a solvent and fixative, which may decalcify early mineralized bone. The omission of Alcian blue steps results in a greater affinity for Alizarin red, and thus provides a more accurate indication of the earliest stages of ossification. To facilitate a more rapid penetration of reagents and reduce superimposing of skeletal elements, it is often beneficial to segment the embryo transversely into head, thorax, pelvis, and tail regions. The head may be further segmented sagittally (hemisectioned) to assist in the visualization of deeper elements of the developing skull (e.g., palatine, vomer). Once hemisectioned, one half of the head can be single stained with Alizarin red while the other half is double stained with Alizarin red and Alcian blue. Opaque (Alizarin red-negative) condensations are often easier to visualize against the Alcian blue-positive endoskeleton of double-stained materials.

1.6 Proliferating Cell Nuclear Antigen (PCNA)-Embedded Serial Section Immunohistochemistry (IHC)

Proliferating cell nuclear antigen (PCNA, also known as cyclin) is a 36 kD protein found within the cell nucleus. It is an auxiliary protein of DNA polymerase delta that is expressed during the S phase of the cell cycle and plays an important role in cell proliferation [33]. We have utilized PCNA immunohistochemistry to identify cell proliferation in American alligator embryos (Fig. 4a: *Alligator mississippiensis*) and postnatal leopard geckos (Fig. 4b: *Eublepharis macularius*).

1.7 Vimentin and Proliferating Cell Nuclear Antigen (PCNA)-Embedded Serial Section Immunofluorescence Double Labeling (IF)

Proliferating cell nuclear antigen (PCNA; as described above) plays an important role in cell proliferation [33]. Vimentin is a 52 kD intermediate filament protein expressed by mesenchymal cells [34], where its major roles include mediating cellular adhesion, migration, and signaling [34]. We have utilized vimentin immunohistochemistry to identify mesenchymal-like/fibroblast-like cells in American alligator embryos (Fig. 5a: *Alligator mississippiensis*) and postnatal leopard geckos (Fig. 5b; *Eublepharis macularius*).

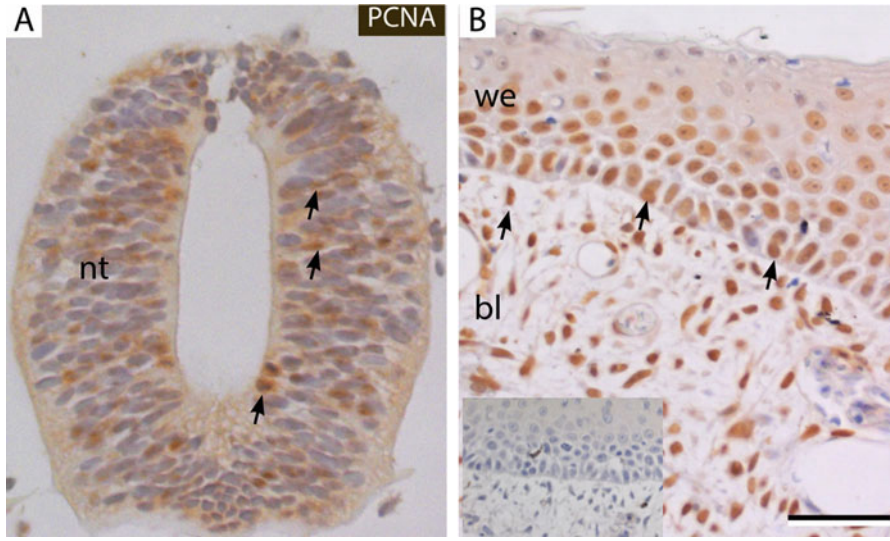

Fig. 4 Proliferating cell nuclear antigen (PCNA) immunohistochemistry in (**a**) the neural tube of a stage 14 American alligator embryo and (**b**) the wound epithelium and blastema of a regenerating leopard gecko tail. Inset in (**b**) is the omission control. Arrows = positive cells, bl = blastema, nt = neural tube, we = wound epithelium. Scale bar = 10 μm

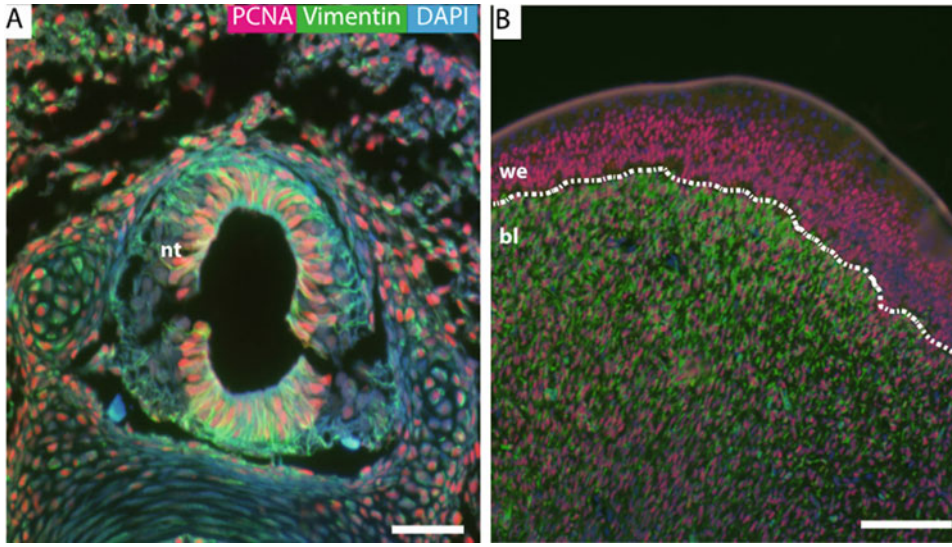

Fig. 5 Proliferating cell nuclear antigen (PCNA) and vimentin immunofluorescence double labeling in (**a**) the neural tube of a stage 21 American alligator embryo and (**b**) the wound epithelium and blastema of a regenerating leopard gecko tail. *Bl* blastema, *nt* neural tube, *we* wound epithelium. Scale bar, **a** = 10 μm, **b** = 20 μm

1.8 Bromodeoxyuridine (BrdU) Injection and Detection Using Immunofluorescence (IF)

BrdU is a thymidine analog that can be incorporated into DNA during the synthesis phase of the cell cycle [35, 36]. We have used a BrdU pulse-chase paradigm (Fig. 6a, b) to document slow-cycling cells across a range of organs (e.g., brain, spinal cord, heart, gut). In these studies, animals receive twice-daily injections of BrdU (50 mg/kg intraperitoneal injection; Fig. 6a) for 7 days (pulse), and are followed for an additional 140 days (chase)[37]. Dividing cells gradually dilutes out the label (~3–4 cell cycles), so those that cycle rapidly can only be detected for short periods of time. At 140 days post-pulse, the only cells retaining the BrdU label are those that cycle slowly. BrdU incorporation can be subsequently visualized using a specialized immunofluorescence protocol (Fig. 6c–f).

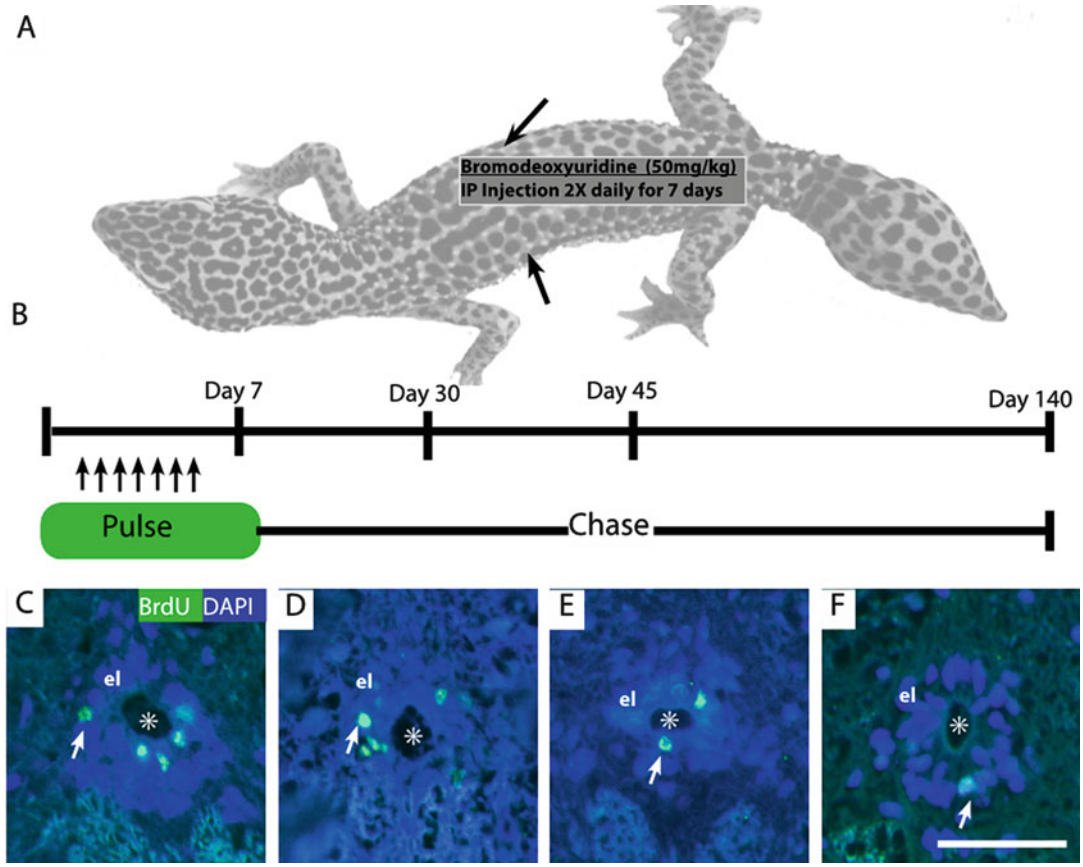

Fig. 6 Bromodeoxyuridine (BrdU) pulse-chase experiment to detect slow-cycling cells in the leopard geckos, *Eublepharis macularius*. (**a**) Intraperitoneal (IP) injections of BrdU (50 mg/kg) are performed twice daily for 7 days. (**b**) Following a 7-day pulse, a selected range of chase periods allow for the assessment of slow-cycling cells. Here we use 30 and 45 days as intermediate time points and 140 days as our long-term chase time point. BrdU immuno-positive cells can be detected using a specialized immunofluorescence protocol and are detected at (**c**) 0 days, (**d**) 30 days, (**e**) 45 days, and (**f**) 140 days following pulse. Asterisk = central canal of spinal cord. Scale bar = 10 μm. Figure adapted from [37]

2 Materials

2.1 Leopard Gecko Husbandry and Breeding

1. Opaque nalgene or polycarbonate enclosures (boxes), ~23 cm × 42 cm × 19 cm high.
2. 1/8″ Thick perforated polyvinyl chloride (PVC) sheeting with staggered ¼″ holes or stainless steel mouse cage lid, bar spacing 1 cm.
3. Water bowls, polycarbonate.
4. Hide boxes (polypropylene food containers cut longitudinally to create two hide boxes).
5. Newspaper, sheets.
6. Mealworms (*Tenebrio molitor*) or crickets (*Acheta domestica* or *Gryllus bimaculatus*).
7. Weigh boat, polypropylene.
8. Powdered calcium and vitamin D3 (cholecalciferol) supplement.
9. Reptile heat cable.
10. Vermiculite.
11. Thermal airflow incubator.

2.2 Transcardial Perfusion for Whole-Animal Fixation

1. Leopard geckos, *Eublepharis macularius*.
2. Ethyl 3-aminobenzoate methanesulfonate salt (MS222).
3. Distilled water (dH_2O).
4. Sodium bicarbonate.
5. Shoebox (or other dark housing unit for leopard gecko).
6. pH test strips.
7. Blood collection set tubing.
8. 60 mL Syringe with luer-lock tip.
9. Instruments for cutting including forceps, scissors, and scalpels.
10. Phosphate-buffered saline (PBS), 10×.
11. 10% Neutral buffered formalin (NBF).
12. 50 mL Centrifuge tubes.
13. 70% Ethanol.

2.3 Postnatal Anesthesia Using Alfaxan (Alfaxalone)

1. Leopard geckos, *Eublepharis macularius*.
2. Alfaxan (Alfaxalone; Abbott Laboratories).
3. Sterile sodium chloride (NaCl).
4. 0.5 cc Insulin syringe.

2.4 Full-Thickness Cutaneous Biopsy Punches

1. Leopard geckos, *Eublepharis macularius.*
2. Alfaxan (Alfaxalone; Abbott Laboratories).
3. Nontoxic permanent marker.
4. Light source.
5. 3 mm Biopsy tool.
6. Tissue forceps, number 11 scalpel blade.

2.5 Whole-Mount Histochemistry: Single (Alizarin Red) and Double (Alizarin Red and Alcian Blue) Embryo Staining Protocol

1. 10% Neutral buffered formalin (NBF).
2. Ethanol (EtOH).
3. Alcian blue 8GX solution: 20 mg Alcian blue 8GX, 70 mL absolute EtOH, 30 mL glacial acetic acid.
4. Glacial acetic acid.
5. Alizarin red S solution: Alizarin red S monohydrate saturated in 0.5% KOH.
6. Distilled water (dH_2O).
7. Trypsin.
8. 2% Sodium borate (NaB_4O_7).
9. 0.5% Aqueous potassium hydroxide (KOH).
10. Glycerol.
11. Slotted spoon.
12. Instruments for cutting and eviscerating, including forceps and scalpel.
13. Glass (or clear) receptacles for fixing, staining, digesting, and storage.

2.6 Paraffin-embedded Serial Section Immunohistochemistry (IHC)

1. American alligator embryos, *Alligator mississippiensis.*
2. Leopard geckos, *Eublepharis macularius.*
3. 10% Neutral buffered formalin (NBF).
4. 28 or 30 gauge needles and syringes.
5. Distilled water (dH_2O).
6. Tissue-decalcifying solution.
7. Automated tissue processor.
8. Formulated purified paraffin synthetic polymer.
9. Tissue cassettes.
10. Tissue forceps and scalpel.
11. Tissue embedding station/console.
12. Rotary microtome.
13. Disposable microtome blades.
14. Gelatin type B.

15. Water bath.
16. Precleaned adhesive or positively charged glass slides.
17. Incubator, preset to 37 °C.
18. Xylene, histological grade.
19. 2-Propanol (= isopropyl alcohol), 70% (w/v) and 100% (w/v).
20. 30% Hydrogen peroxide (H_2O_2).
21. Staining boat and staining dishes.
22. Phosphate-buffered saline (PBS), 10×.
23. Coplin jars.
24. Humidity chamber.
25. Antistatic/lint-free wipes.
26. 100–1000 μL Pipette and general-purpose 101–1250 μL pipette tips.
27. 10–100 μL Pipette and general-purpose 1–200 μL pipette tips.
28. 0.5–10 μL Pipette and general-purpose 0.1–10 μL pipette tips.
29. Blocking solution: 3% Normal goat serum in PBS.
30. PCNA rabbit polyclonal IgG: 1:500 dilution in PBS (Proteintech, 10205-2-AP).
31. Biotin-SP-conjugated AffiniPre F(ab')2 Fragment Goat Anti-Rabbit IgG (H + L): 1:1000 dilution in PBS (Vector Laboratories, BA-9200).
32. Peroxidase-conjugated streptavidin: 1:200 Dilution (Jackson ImmunoResearch Laboratories, 016-030-084).
33. 3,3′-Diaminobenzidine (DAB) (Peroxidase Substrate Kit, Vector Laboratories, SK-4100).
34. Harris modified hematoxylin.
35. Ammonia water: 0.25% Ammonium hydroxide in dH_2O.
36. Microscope slide mounting medium.
37. Microscope cover glass, 22 mm × 50 mm × 1 mm.

2.7 Paraffin-embedded Serial Section Double-Label Immunofluorescence (IF)

1. American alligator embryos, *Alligator mississippiensis*.
2. Leopard geckos, *Eublepharis macularius*.
3. 10% Neutral buffered formalin (NBF).
4. 28 or 30 gauge needles and syringes.
5. Distilled water (dH_2O).
6. Cal-Ex decalcifier solution.
7. Automated tissue processor.
8. Formulated purified paraffin synthetic polymer.
9. Tissue cassettes.

10. Tissue forceps and scalpel.
11. Tissue embedding station/console.
12. Rotary microtome.
13. Disposable microtome blade.
14. Gelatin type B.
15. Water bath.
16. Precleaned adhesive or positively charged glass slides.
17. Incubator preset to 37 °C.
18. Xylene, histological grade.
19. 2-Propanol (= isopropyl alcohol), 70% (w/v) and 100% (w/v).
20. Staining boat and staining dishes.
21. Phosphate-buffered saline (PBS), 10×.
22. Coplin jars.
23. Humidity chamber.
24. Antistatic/lint-free wipes.
25. 100–1000 μL Pipette and general-purpose 101–1250 μL pipette tips.
26. 10–100 μL Pipette and general-purpose 1–200 μL pipette tips.
27. 0.5–10 μL Pipette and general-purpose 0.1–10 μL pipette tips.
28. Blocking solution: 5% Normal goat serum in PBS.
29. PCNA rabbit polyclonal IgG: 1:100 dilution in PBS (Proteintech, 10205-2-AP).
30. Vimentin mouse monoclonal IgG1: 1:50 dilution in PBS (Developmental Studies Hybridoma Bank, H5).
31. Goat anti-mouse Alexa Fluor 488 IgG: 1:200 dilution in PBS (Life Technologies, A11001).
32. Goat anti-rabbit Cy3-conjugated IgG: 1:200 dilution in PBS (Jackson ImmunoResearch Laboratories, 111-165-144).
33. 4′,6′-Diamidino-2-phenylidole, dihydrochloride (DAPI) counterstain: 1:10000 dilution in PBS (Thermo Fisher Scientific, D1306).
34. Fluorescent mounting media.
35. Microscope cover glass, 22 mm × 50 mm × 1 mm.

2.8 Bromodeoxyuridine (BrdU) Injection and Immunodetection

1. Leopard geckos, *Eublepharis macularius*.
2. Bromodeoxyuridine (BrdU) powder (Sigma-Aldrich, B5002).
3. Dimethyl sulfoxide.
4. Sterile 1× PBS.
5. 0.5 cc Insulin syringes.

6. 10% Neutral buffered formalin (NBF).
7. 28 or 30 gauge needles and syringes.
8. Distilled water (dH_2O).
9. Tissue-decalcifying solution.
10. Xylene, histological grade XP3-1GAL.
11. 2-Propanol (= isopropyl alcohol), 70% (w/v) and 100% (w/v).
12. Staining boat and staining dishes.
13. Phosphate-buffered saline (PBS), 10×.
14. Coplin jars.
15. Humidity chamber.
16. Antistatic/lint-free wipes.
17. 100–1000 μL Pipette and general-purpose 101–1250 μL pipette tips.
18. 10–100 μL Pipette and general-purpose 1–200 μL pipette tips.
19. 0.5–10 μL Pipette and general-purpose 0.1–10 μL pipette tips.
20. Trypsin.
21. Bovine serum albumin.
22. TWEEN 20 (Sigma, P5927).
23. Sodium azide.
24. 2 N Hydrochloric acid.
25. Blocking solution: 5% Normal goat serum (Vector Laboratories, S1000) in PBS.
26. Anti-bromodeoxyuridine, mouse monoclonal IgG: 1:100 dilution (Sigma-Aldrich, B8434).
27. Goat anti-mouse Alexa Fluor 488 IgG: 1:200 dilution in PBS (Life Technologies, A11001).
28. 4′,6′-Diamidino-2-phenylidole, dihydrochloride (DAPI) counterstain: 1:10000 dilution in PBS (Thermo Fisher Scientific, D1306).
29. Fluorescent mounting media (Dako, S3023).
30. Microscope cover glass, 22 mm × 50 mm × 1 mm.

3 Methods

3.1 Leopard Gecko Husbandry and Breeding

As for most reptiles, mating is stimulated by changes in photoperiod and temperature. Reproductive success is enhanced by cycling both males and females. Cycling involves a period of dormancy (summarized in [20]).

1. Reduce photoperiod by 0.5 h every second day until it reaches 8-h light:16-h dark. During the dark hours, reduce ambient

temperature to 21 °C and turn off the heat gradient. Reduce the amount of food being offered.

2. Once a 8:16 photoperiod is reached, suspend feeding and maintain the ambient temperature at a near-constant 21 °C with no heat gradient. Freshwater should always be made available.
3. After 2 months, reverse the photoperiod back to 12:12, at 0.5-h increments every second day, and return the ambient temperature to 22–32 °C. Resume daily feeding regime.
4. To initiate breeding, a single male can be introduced to one or more females for a period of 1–5 days. Females are capable of storing sperm [38], and may produce multiple clutches within a single year-long season. Successful mating will result in a clutch of two eggs every 21–28 days. Oviposition occurs approximately 11 days after ovulation [39].

3.2 Transcardial Perfusion for Fixation of Whole Embryonic and Adult Leopard Geckos

1. Prepare MS222 stock solution by adding 0.1 g MS222 powder to 100 μL dH_2O and vortex until dissolved.
2. Prepare MS222 working solution by adding 2–3 drops of the stock solution to 750 μL of dH_2O.
3. Test pH using test strips and balance pH to 7.0 using sodium bicarbonate powder mixed directly into the tube.
4. Inject full volume of working solution in a divided dose into the intraperitoneal cavity on either side of the heart (*see* **Note 4**).
5. Following loss of righting reflex and muscle tone, inject the remaining volume of the stock solution into the intraperitoneal cavity.
6. Following chemical euthanasia, make a horizontal mid-thoracic incision and two lateral incisions from the mid-thoracic ribcage to the clavicle using scissors and forceps to expose the heart.
7. Using the blood collection tubing and needle, insert the needle into the apex of the ventricle and attach a 60 mL syringe, filled with PBS.
8. Slowly depress the syringe until all blood products have been flushed (*see* **Note 5**).
9. Remove the syringe filled with PBS from the blood collection tubing, switch to a second 60 mL syringe filled with 10% NBF, and slowly depress the syringe until the gecko is fully perfused (*see* **Note 6**).
10. Following perfusion, dissect out the tissue of interest and place in 10% NBF for 24 h.
11. Rinse with dH_2O.
12. Store in 70% ethanol until tissue is processed.

3.3 Anesthesia for Postnatal Leopard Geckos Using Alfaxan (Alfaxalone)

1. Weigh geckos to determine the volume of Alfaxan required (30 mg/kg) for each injection.
2. Dilute Alfaxan (2 mg/mL) in sterile 0.9% NaCl.
3. Draw volume into a 0.5 cc insulin syringe.
4. Manually restrain gecko and inject Alfaxan in a divided dose into epaxial muscle mass on either side of the body midline (*see* **Note 7**).

3.4 Full-Thickness Cutaneous Biopsy Punches in Postnatal Leopard Geckos

1. Anesthetize leopard gecko (*see* Subheading 3.3).
2. Mark the site of the biopsy using a nontoxic permanent marker (*see* **Note 8**).
3. Stretch the skin surrounding the biopsy location until taut.
4. Center the biopsy punch perpendicularly over the chosen site and twist slowly several times in each direction until the epidermis is punctured around the circumference of the tool.
5. Use tweezers, forceps, and scalpel blade to remove the plug of tissue from the wound site.

3.5 Whole-Mount Histochemistry: Single (Alizarin Red) and Double (Alizarin Red and Alcian Blue) Embryo Staining Protocol (Modified From [25])

All steps take place at room temperature.

1. Fix embryos in 10% NBF for 24 h (*see* Subheading 3.2) (*see* **Note 9**).
2. Briefly rinse with dH_2O.
3. Transversely segment specimen into head and one or more body regions (e.g., pectoral and pelvic regions) and eviscerate (*see* **Note 10**). Sagittally section (hemisection) head into left and right halves. Skinning embryos is usually not necessary.
4. If single staining with Alizarin red S skip to 7. Otherwise, rinse with dH_2O.
5. Stain with Alcian blue 8GX solution overnight.
6. Hydrate through an EtOH series for a minimum of 1 h at each step in the series. Begin with two changes of absolute EtOH, and then 95%, 70%, 40%, 15%, and finally into dH_2O.
7. Macerate with either 1% trypsin in a 2% sodium borate solution (for larger specimens or those in the later stages of development with more fully differentiated tissues present) or 0.5% KOH (for smaller, delicate, or early-staged specimens). Check every 2–6 h until specimen becomes limp (*see* **Note 11**). If necessary, place specimen in dH_2O overnight and then replace into fresh maceration solution.
8. Stain with Alizarin red S solution for 24 h (*see* **Note 12**).
9. Clear for 24 h at each stage of a 0.5% KOH–glycerol series beginning with 3:1, 1:1, 1:3, and then pure glycerol (*see* **Note 13**).
10. Store in glycerol (*see* **Note 14**).

3.6 Paraffin-Embedded Serial Section Immunohistochemistry (IHC)

1. Fix embryos in 10% NBF for 24 h (*see* Subheading 3.2) (*see* **Note 15**) .
2. Rinse with dH_2O.
3. Store in 70% EtOH.
4. Dissect the tissue of interest (e.g., limbs) from embryos using a scalpel or fine dissection tools and place into tissue cassettes. Later staged embryos (e.g., Ferguson stage 17 or later [13]) should be decalcified for 10 minutes in decalcifying solution and briefly rinsed with dH2O prior to tissue processing.
5. Dehydrate and then infiltrate tissues with molten paraffin wax (*see* **Note 16**).
6. Embed tissues in paraffin wax and allow blocks to cool.
7. Cool tissue blocks on ice for 30 min prior to sectioning. Section blocks into slices 5 mm thick using a rotary microtome.
8. Transfer sections to a water bath, mount on positively charged slides, and dry overnight in an incubator preset to 37 °C.
9. Deparaffinize slides using three changes of xylene (2 min each).
10. Rehydrate through three changes of 100% 2-propanol, 70% 2-propanol, and dH_2O (2 min each).
11. Quench slides in 1.0% H_2O_2 for 10 min, and rinse in dH_2O and three changes of PBS (2 min each).
12. Remove excess fluid from slide by wiping around the tissue sample with an antistatic/lint-free wipe.
13. Apply 150 μL of blocking solution to each tissue sample and incubate slides for 1 h at room temperature in a humidified chamber.
14. Tip off blocking solution and apply 150 μL of primary antibody (1:500 dilution) to each test slide. Apply sterile PBS to the negative control. Incubate slides at 4 °C overnight in a humidified chamber.
15. Rinse slides in three changes of PBS (2 min each) (*see* **Note 17**). Remove excess liquid from slides using antistatic/lint-free wipes. Apply 150 μL biotinylated secondary antibody (1:1000 dilution) to each tissue sample and incubate slides for 1 h in a humidified chamber at room temperature.
16. Rinse slides in three changes of PBS (2 min each) (*see* **Note 17**). Remove excess liquid from slides using antistatic/lint-free wipes. Apply 150 μL streptavidin HRP (1:200 dilution) to each tissue sample and incubate slides for 1 h in a humidified chamber at room temperature.
17. Rinse slides in three changes of PBS (2 min each) (*see* **Note 17**). Submerge slides in a DAB solution for 40 s and then rinse under running water.

18. Counterstain with Harris hematoxylin (2 dips), rinse in running water, and blue in ammonia water (*see* **Note 18**).
19. Dehydrate through three changes of clean 100% 2-propanol (2 min each) and three changes of xylene (2 min each). Add coverslip using cytoseal mounting solution.

3.7 Paraffin-Embedded Serial Section Double-Label Immunofluorescence (IF)

1. Fix embryos in 10% NBF for 24 h (*see* Subheading 3.2) (*see* **Note 15**).
2. Briefly rinse with dH_2O.
3. Store in 70% EtOH.
4. Dissect the tissue of interest (e.g., limbs) from embryos using a scalpel or fine dissection tools and place into tissue cassettes. Later staged embryos (e.g., Ferguson stage 17 or later [13]) should be decalcified for 10 min in decalcifying solution and briefly rinsed with dH_2O prior to tissue processing. For postnatal gecko tissue-containing bone, tissue should be decalcified for 30 min in decalcifying solution and briefly rinsed with dH_2O prior to tissue processing.
5. Dehydrate and then infiltrate tissues with molten paraffin wax (*see* **Note 16**).
6. Embed tissues in paraffin wax and allow blocks to cool.
7. Cool tissue blocks on ice for 30 min prior to sectioning. Section blocks into slices 5 mm thick using a rotary microtome.
8. Transfer sections to a water bath, mount on positively charged slides, and dry overnight in an incubator preset to 37 °C.
9. Deparaffinize slides using three changes of xylene (2 min each).
10. Rehydrate through three changes of 100% 2-propanol, 70% 2-propanol, and dH_2O (2 min each).
11. Rinse slides using three changes of PBS (5 min each).
12. Remove excess fluid from slide by wiping around the tissue sample with an antistatic/lint-free wipe.
13. Apply 150 μL of blocking solution to each tissue sample and incubate slides for 1 h at room temperature in a humidified chamber.
14. Tip off blocking solution and apply 150 μL of primary antibodies (1:100 dilution for PCNA; 1:50 dilution for vimentin) to each test slide. Apply sterile PBS to the negative control. Incubate slides at 4 °C overnight in a humidified chamber.
15. Rinse slides in three changes of PBS (5 min each) (*see* **Note 17**). Remove excess liquid from slides using antistatic/lint-free wipes. Apply 150 μL fluorescent-conjugated secondary antibodies (Cy3 (goat anti-rabbit) 1:200; Alexa488 (goat anti-mouse) 1:200) to each tissue sample and incubate slides for 1 h in a humidified chamber at room temperature (*see* **Note 19**).

16. Rinse slides in three changes of PBS (5 min each) (*see* **Note 17**). Remove excess liquid from slides using antistatic/lint-free wipes.
17. Apply 150 μL of DAPI counterstain (1:10000 dilution) to each tissue sample and incubate slides for 5 min in a humidified chamber at room temperature.
18. Rinse slides in three changes of PBS (5 min each) (*see* **Note 17**).
19. Add coverslip using fluorescent mounting media.

3.8 Bromodeoxyuridine (BrdU) Injection and Immunodetection

1. Weigh geckos to determine the volume of BrdU required (50 mg/kg) for each injection.
2. Inject BrdU into the intraperitoneal cavity, just caudal to the forelimbs using a 0.5 cc insulin syringe (*see* **Note 20**), every 12 h for the duration of the 7-day pulse period.
3. Fix postnatal tissue using transcardial perfusion (*see* Subheading 3.2), followed by 24-h 10% NBF emersion fixation on a shaker.
4. Rinse with dH_2O.
5. Store in 70% EtOH.
6. Dissect the tissue of interest (e.g., limbs) from embryos using a scalpel or fine dissection tools and place into tissue cassettes. For tissue-containing bone, tissue should be decalcified for 30 min in decalcifying solution and briefly rinsed with dH_2O prior to tissue processing.
7. Dehydrate and then infiltrate tissues with molten paraffin wax (*see* **Note 16**).
8. Embed tissues in paraffin wax and allow blocks to cool.
9. Cool tissue blocks on ice for 30 min prior to sectioning. Section blocks into slices 5 mm thick using a rotary microtome.
10. Transfer sections to a water bath, mount on positively charged slides, and dry overnight in an incubator preset to 37 °C.
11. Deparaffinize slides using three changes of xylene (2 min each).
12. Rehydrate through three changes of 100% 2-propanol, 70% 2-propanol, and dH_2O (2 min each).
13. Rinse slides using one rinse of PBS (15 min).
14. Remove excess fluid from slide by wiping around the tissue sample with an antistatic/lint-free wipe (*see* **Note 17**).
15. Apply 150 μL of 2 N HCl to each tissue sample and incubate slides for 30 min at 37 °C in a humidified chamber (*see* **Note 21**).
16. Rinse slides using one rinse of PBS (2 min).

17. Apply 150 μL of 0.1% trypsin to each tissue sample and incubate slides for 20 min at 37 °C in a humidified chamber (*see* **Note 21**).
18. Rinse slides using one rinse of PBS (2 min).
19. Apply 150 μL of blocking solution (5% NGS in diluent) to each tissue sample and incubate slides for 30 h at 37 °C in a humidified chamber (*see* **Note 21**).
20. Tip off blocking solution and apply 150 μL of primary antibodies (1:100 dilution for BrdU) to each test slide. Apply sterile PBS to the negative control. Incubate slides at 37 °C for 2 h in a humidified chamber.
21. Rinse slides using three changes of PBS (2 min each) (*see* **Note 17**).
22. Apply 150 μL fluorescent-conjugated secondary antibody (Alexa488 (goat anti-mouse) 1:200) to each tissue sample and incubate slides for 1 h in a humidified chamber at room temperature (*see* **Note 19**).
23. Rinse slides using three changes of PBS (2 min each) (*see* **Note 17**).
24. Apply 150 μL of DAPI counterstain (1:10000 dilution) to each tissue sample and incubate slides for 5 min in a humidified chamber at room temperature.
25. Rinse slides in three changes of PBS (2 min each) (*see* **Note 17**).
26. Add coverslip using fluorescent mounting media.

4 Notes

1. Although details of reptile husbandry are taxonomically variable and often species specific [40], the basic environmental factors to consider include the following:

   Temperature—reptiles are poikilothermic and require a temperature gradient in order to thermoregulate. Daily and seasonal temperature fluctuations, and even a period of hibernation (brumation), may be necessary to stimulate breeding. Preferred temperature ranges for many common pet-trade reptiles are provided by Rossi [40]. Temperature gradients for terrestrial species can be established using subsurface heating sources (e.g., heating pads), whereas arboreal species may require the use of radiant heat sources such as ceramic heaters and heat lamps. Use of a rheostat and thermometer is beneficial to maintaining constant temperatures.

Photoperiod and ultraviolet light—photoperiod (amount of daylight) is also an important external stimulus for initiating breeding, particularly for temperate and subtropic species. In addition, many reptiles require exposure to ultraviolet B (UVB) spectrum radiation (290–320 nm) to promote vitamin D3 synthesis. Vitamin D3 is then used to absorb calcium. For species that are active at dawn/dusk, vitamin D3 powdered supplement may be used instead of a UVB light source.

Humidity—humidity requirements vary with the species. Saturated environments with poor ventilation will promote fungal and bacterial growth that can lead to disease. Artificial cover (e.g., plastic hide boxes) may be used to provide microenvironments of elevated humidity for incubating eggs or during periods of skin shedding (ecdysis). Positioning a water dish above a subsurface heat source will provide a localized increase in humidity. Well-ventilated enclosures may require daily misting.

Substrate—in laboratory settings, inexpensive and easy-to-replace substrates such as newspaper have obvious advantages. However, some species prefer to create burrows and/or deposit eggs in soil/sand. Therefore details of biology and captive management should be investigated prior to establishing a breeding colony. Although granular substrates may have aesthetic appeal and be easier to establish an elevated humidity, in many small- and medium-size species ingested particles can lead to gastrointestinal impaction and death. Aromatic substrates like cedar bark can lead to irritation and death due to secondary complications [40] and should be avoided.

Diet and water—reptiles include a large array of carnivores, insectivores, herbivores, and diets in between. Nutritional requirements based on observations in the wild should be investigated if data are available. The popularity of many reptiles as pets has made many captive-raised prey including mice, rats, and arthropods widely accessible year round. Arthropods are typically low in calcium and should be raised on diets of calcium-rich leafy greens ("gut loaded") [41]. It may also be necessary to dust arthropods with powdered calcium supplement prior to being offered. Free access to freshwater is usually necessary, although species from arid climates may prefer occasional misting. Terrestrial species typically make use of floor-based water dishes whereas arboreal species may require misting, a drip system, or an elevated water source.

Enclosure—minimal enclosure floor space and height will depend on the size and behavior of the species [40]. Consideration should be given to social factors such as intraspecific aggression and territorial behaviors. Opaque enclosures or

opaque cage dividers may be necessary. Aquatic species (crocodilians, some turtles) require a terrestrial area to haul out of the water and bask. Enclosures, water bowls, hide boxes, and other cage furniture must be disinfected on a regular basis using either a standard cage washing machine or a disinfection solution such as sodium hypochlorite (household bleach, 2–10% solution). It should be noted that some captive-bred and wild reptiles (including common pet-trade species such as leopard geckos and Eastern corn snakes) may be infected with *Cryptosporidium* sp., a coccidian protozoan that produces oocysts resistant to disinfection by bleach. Cryptosporidiosis can lead to chronic weight loss, diarrhea, lethargy, and death. To avoid cross-contamination, enclosures housing individuals suspected of having cryptosporidiosis should be disinfected with ammonia water (5% solution) followed by a period of air-drying [42].

2. Sex determination in reptiles may be the result of genetic or environmental factors. In genotypic sex determination, sex chromosomes determine males from females. Genotypic sex determination includes male heterogamety, in which males are characterized by having two dissimilar sex chromosomes; female heterogamety, in which females are characterized by having two dissimilar sex chromosomes; and genetic systems that have not been linked to heteromorphic sex chromosomes [43]. Various turtles and lizards, as well as most snakes, use genetic sex determination.

   Alternatively, in all crocodilians and numerous species of turtles and lizards [43], there are no sex chromosomes. Instead, sexual identity is established during embryogenesis by incubation temperature [43]. It is worth noting that the effects of incubation temperature are cumulative in both magnitude and duration [44, 45]. The use of species that employ temperature-dependent sex determination (TSD) in experimental settings has many obvious advantages and it is often possible to produce a 1:1 sex ratio at pivotal species-specific temperatures [46]. The specific timeframe during which eggs are sensitive to incubation temperature has been established for several species, typically within the middle third to half of embryonic development [46, 47]. Gonadal differentiation has yet to occur when this period begins, but by the conclusion of this period sex-specific gonadal changes have become apparent [46].

   As with genotypic sex determination, there are several distinctive patterns of TSD. Crocodilians and some lizards employ female-male TSD, wherein lower incubation

temperatures result in a predominately female population and higher incubation temperatures result in a predominately male population [48]. Conversely, male-female sex determination occurs in many Testudines. Another pattern of TSD is female-male-female found in some crocodilians, lizards, and Testudines. In female-male-female TSD, predominately female populations are produced at lower and higher temperatures, whereas males are produced at intermediate temperatures [48]. Lastly, there are some species of the gecko *Tarentola* in which males are produced at lower temperatures, females are produced at intermediate temperatures, and a balanced sex ratio is found at high temperatures [49].

3. Reptile eggs are categorized as either hard-shelled or with parchment-like (leathery; soft) shells [50]. Both types have an inorganic outer calcareous layer, primarily composed of calcium carbonate [51], and an organic inner layer or shell membrane. The thickness and continuity of the calcareous layer determine the overall structure of the eggshell. Shells with a thick and continuous calcareous layer are "hard" whereas those with a thin and/or discontinuous calcareous layer are parchment-like [50]. Discontinuous calcareous layers allow for more flexibility and expansion due to embryo growth, but are consequently more difficult to window for in ovo manipulations [51].

   The shell membrane lies deep to the calcareous layer and consists of multiple fibrous sublayers or horizons [52]. Fibers within each horizon demonstrate differing orientations [50] and the exact number of fibrous horizons is taxonomically variable. The deepest horizon of the shell membrane is the amorphous layer (= inner boundary layer [53] *or* limiting membrane [54]). This amorphous layer has many small pores but is considered to function as a barrier to pathogens during development [55].

4. MS222 chemical euthanasia can be used for leopard geckos up to 30 g in mass. For geckos above this weight, MS222 is less effective and an overdose of Alfaxan should be used. In geckos ranging from 30 to 60 g, 150 μL of concentrated Alfaxan in a divided dose as described in Subheading 3.3 is advised.
5. Approximately 4 mL of PBS per gram of should be flushed through the gecko until blood products are no longer exiting. Fluid should be pushed into the heart slowly and carefully to preserve the vasculature.
6. Approximately 4 mL of 10% NBF per gram should be used to fix the gecko; fixation can be evaluated by checking the stiffness of the limbs; if 4 mL/g is not sufficient, more fixative can be

used. Limbs should be fully stiff following transcardial perfusion.

7. Geckos should be restrained by grasping bilaterally using your thumb and index finger just rostral to the forelimbs. Anesthetic plane can be assessed by confirming (1) loss of righting reflex by positioning geckos on their dorsal surface and assessing their ability to right themselves and by (2) evaluating their withdrawal response following a toe pinch. Without anesthesia, geckos can rapidly right themselves when inverted and will withdraw their toe when pinched yet under complete anesthesia cannot right themselves and will not withdraw their toe.
8. When choosing a site to biopsy, we have found that centering on a large tubercle scale (*see* Fig. 3) allows for clear visualization of the open wound, healing wound, and fully healed wound.
9. Specimens for whole-mount histochemistry can be stored in 70% EtOH at room temperature for 1 h to months (or even years) prior to staining. Transfer from NBF to 70% EtOH should be done using forceps. Tissues need to be rehydrated through a graded EtOH series (40%, 15%, dH_2O) for 2 min each prior to staining.
10. Segmented specimens are easier to completely eviscerate. It is worth noting that Testudines, crocodilians, and *Sphenodon* have skeletal structure located superficially across the abdomen (plastral elements, gastralia). In order to maintain these elements in situ across the ventral midline, avoid opening the abdominal cavity with a sagittal incision. Alternatively, remove the viscera by making an incision in the lateral body wall (through the rib elements) on one side of the abdomen. Hemisectioning heads permits internal or deep skeletal elements (e.g., bones of the palate) to be visualized.
11. Following maceration specimens will be very delicate. Use a slotted spoon to transfer specimens into subsequent solutions.
12. Alizarin red S stains for structures mineralized with calcium salts. Most commonly this is bone but may also include calcified cartilage, dental tissues, and calcified endolymphatic ducts. These ducts may be very prominent in the occipital area of the head among early-staged reptile embryos. Premineralized bone (osteoid) condensations will be Alizarin red negative. Prior to calcification osteoid condensations are visible using transmitted light as white or opaque weblike concentrations of tissue.
13. Make stock solutions of the 0.5% KOH–glycerol series ahead of time to insure that they are well mixed and without bubbles.

14. Prior to photography, position the specimen and wait for 1–3 h to reduce distortions in the glycerol. Substage illumination highlights the in situ organization of the skeleton. For small specimens, it may be necessary to image using a dissection microscope.
15. Embryonic specimens must be staged prior to processing using an appropriate staging table. If possible, stage prior to fixation or soon thereafter to minimize distortion of tissues and color change. Ferguson [14] provides a detailed embryonic staging table for American alligator. Avoid over-fixation (i.e., longer than 24 h). Specimens fixed with 10% NBF do not necessarily require antigen retrieval. Enzymatic digestion (e.g., pronase) and heat-induced epitope retrieval (citrate buffer at 80 °C) may result in increased amounts of background staining compared with samples that did not undergo retrieval.
16. Paraffin wax infiltration may be accomplished using an automated tissue processor, or manually. Tissues processed manually must be dehydrated with six changes of 2-propanol (70%, 85%, 90%, 95%, 100%, 100%; 45 min to 1 h each), followed by three changes of xylene (or other organic solvent; 45 min each or until tissues have cleared), and then three changes of molten paraffin wax done under vacuum (45 min to 1 h each). For ease of handling, place tissue samples in tissue cassettes.
17. Unless treated carefully, tissue sections may lift off the slides. Avoid agitating slides and use Coplin jars, rather than a spray bottle, to wash with PBS.
18. The intensity of the background staining can be altered by either increasing or decreasing the number of dips in hematoxylin. In addition, you can decrease the intensity of the hematoxylin after you have stained by dipping in 1% acid alcohol (a 1% solution of hydrochloric acid in 70% alcohol).
19. Following the application of secondary antibodies to tissue samples, immunofluorescence protocols should be performed in dark conditions to preserve fluorescently tagged secondary antibodies and DAPI counterstain. This can be accomplished using low light and incubating samples in a closed box.
20. For BrdU injections we alternated between left- and right-sided injections at each 12-h injection interval. For example, A.M. injection = right side, P.M. injection = left side.
21. For BrdU immunostaining, we placed a humidity chamber, 2 N HCl, 0.1% trypsin, and diluent in a 37 °C oven just prior to the beginning of the immunostaining protocol to ensure that the humidity chamber and all reagents reached 37 °C prior to their application to the tissue. Use a hydrophobic pen to outline tissue samples prior to 2 N HCl application to ensure that solutions stay in place overtop of the tissue sections.

Without hydrophobic circles, HCl, 0.1% trypsin, and diluent will spread across the slide and not stay on the tissue samples.

Acknowledgments

We thank Pat A.D. Wise for comments on the manuscript, Helen Coates and Katie McLean for assistance developing the immunohistochemical protocols, Hanna Peacock for assistance developing the biopsy protocol, and Ilinca Lungu for assistance with the photography. This work is funded by the Natural Sciences and Engineering Research Council of Canada.

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Chapter 14

Manipulation of Developmental Function in Turtles with Notes on Alligators

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Abstract

Reptiles have great taxonomic diversity that is reflected in their morphology, ecology, physiology, modes of reproduction, and development. Interest in comparative and evolutionary developmental biology makes protocols for the study of reptile embryos invaluable resources. The relatively large size, seasonal breeding, and long gestation times of turtles epitomize the challenges faced by the developmental biologist. We describe protocols for the preparation of turtle embryos for ex ovo culture, electroporation, in situ hybridization, and microcomputed tomography. Because these protocols have been adapted and optimized from methods used for frog, chick, and mouse embryos, it is likely that they could be used for other reptilian species. Notes are included for alligator embryos where appropriate.

Key words Turtle, In situ hybridization, Electroporation, Ex ovo culture, Embryology, Microcomputed tomography, Alligator

1 Introduction

The development of reptiles has long been understudied mostly because limited availability and seasonal reproduction make many reptile embryos difficult to obtain in sufficient quantity. Why study developmental genetics in non-model organisms? The investigation of the genetic mechanisms that underlie developmental processes is another line of evidence contributing to the study of biological complexity. The establishment of additional vertebrate systems for use in developmental genetic investigation will provide greater explanatory power to hypotheses about their evolution.

The morphogenesis of the turtle has long been appreciated, as the evolution of the turtle shell has puzzled developmental biologists and systematists [1]. Turtles have also been an important research model in studies on sex determination [2], the nervous system [3], bone formation [4], skin patterning [5], and hypoxia [6].

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_14,

Here, we include protocols to describe morphogenesis and to test molecular function during turtle development. These include various manipulations using ex ovo culture and methods to assay experimental outcomes. Turtle embryo explants can be maintained in a modified organ culture system at the gas:fluid interface on a nucleopore membrane, and continue to develop for up to 3 weeks. Embryos can be cultured at a wide range of stages, as defined by the tables of Greenbaum [7] and Yntema [8]. We have used G10-G15 [7]/Y10–14 [8] embryos for electroporation, G15 (Y14) embryos for experiments manipulating the carapacial ridge (primordium of the turtle shell carapace; [9]), Y15–17 embryos for scute development [5], and G/Y 16–17 embryos for plastron bone differentiation (the plastron is the ventral portion of the turtle shell; [4]).

Fluorescent cell labeling in the turtle embryo for long-term observation of cell movements has also been problematic. This is primarily due to inherent challenges of microinjection of fluorescent dyes and lack of methods to deliver DNA plasmids directly into cells. In vivo electroporation is an efficient technique that has been successfully applied in avian and other embryos and adult model organisms (zebrafish, frog, mouse) to reproducibly fluorescently label cells and/or alter gene expression in single cells and tissues [10–13]. Briefly, DNA constructs are injected into or near a subregion of interest and then electrical pulses are applied to the area. The electrical pulses open temporary pores in cell membranes and allow the injected DNA material to move along the electrical current. DNA is negatively charged and will move toward the positive electrode, thereby into the cell(s) of interest.

We present an electroporation method optimized in turtle embryos to produce high-efficiency transfection of DNA constructs into cells of interest. We overcome the challenges of the fragility of the turtle eggshell and random position of the embryo within the egg by developing an ex ovo electroporation method that is comparable to the efficiency and reproducibility of our experience with avian embryos. Morphological observations are a strong basis for inferring developmental function. To examine when and where gene products regulate morphogenesis, we include a protocol for whole-mount in situ hybridization using digoxigenin-labeled (nonradioactive) probes [5, 14] and one for staining of soft tissue for microcomputed tomography (derived from [15], applied in [5]).

Unlike avian embryos, turtle embryos have rarely been successfully manipulated in ovo. Turtle species that have been successfully manipulated in ovo lay hard-shelled eggs (*Chelydra serpentina*, [16, 17]; *Pelodiscus sinensis*, [18]). Many turtles, however, including the species we use, the slider turtle *Trachemys scripta*, lay flexible-shelled eggs. Freshly laid turtle and alligator eggs can be collected from a commercial turtle or alligator farm, or from the wild. The collection of eggs from a farm makes the assessment of

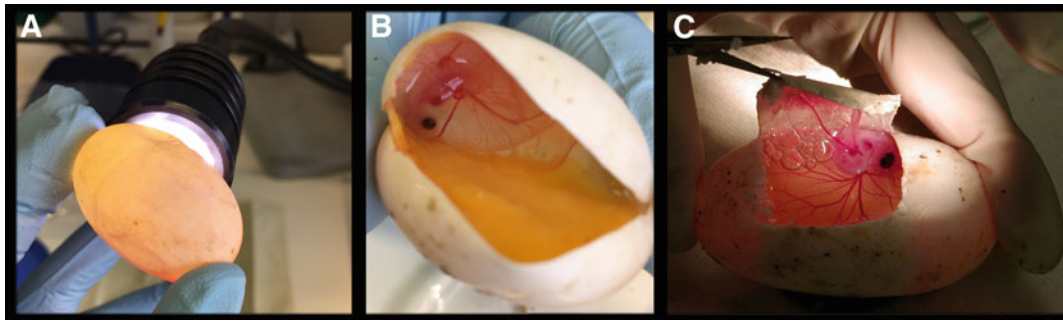

Fig. 1 The embryology of reptiles. (**a**) Candling a turtle egg with oblique light reveals the vitelline circulatory system and position of the embryo. (**b**) An opened slider turtle egg shows the turtle embryo and extraembryonic membranes fused to the inner shell. (**c**) An opened American alligator egg shows the embryo laying dorsal to the yolk, suspended from the inner eggshell in extraembryonic membranes

time of egg laying easier than if they are collected in the wild and allows the harvest of a greater number of eggs. The subspecies *T. scripta elegans* (red-eared slider) is considered a pest and turtle farms generate great numbers of this species for the pet trade.

After the eggs are laid, the extraembryonic membranes of the turtle and alligator embryos fuse with the inner shell membrane of the egg, and the embryo migrates dorsally along these membranes to lie underneath the top of the egg ([19, 20]; Fig. 1). It is, therefore, important to set the eggs and keep them in the same orientation throughout development to prevent the yolk from crushing and killing the embryo. Turtle eggs collected from the wild should be candled or looked at with oblique light to determine the position of the embryo before setting the eggs (Fig. 1). The stage of turtle and alligator embryos can be approximated by the degree of banding on the dorsal surface of the egg [19, 20].

Turtle and alligator eggs are normally transported in moist vermiculite. If turtle eggs are collected immediately after they are laid, they may be transported in hypoxic conditions by vacuum sealing eggs in plastic bags and keeping them cool ([21]; *see* **Note 1**). This hypoxic method of transport cannot be done with crocodilian eggs [22], likely because the embryos are at a more advanced stage of development than turtles when eggs are laid [20].

The eggs of turtles require a humid environment to prevent desiccation. The incubation of eggs in a 1:1 mix of water and vermiculite (w/v) in plastic containers alleviates this problem. The plastic containers are then placed in an incubator to regulate temperature. The containers and their lids should be punctured to allow exchange of air. Set the eggs horizontally and side by side in rows. The vermiculite should be periodically rehydrated because it can ultimately desiccate the collection of eggs. Eggs that contain dead embryos must be removed because these are susceptible to fungal infection and could contaminate other eggs.

Sex determination in *T. scripta* has been shown to depend on temperature [23]. In *T. scripta*, 1:1 ratio of males to females is achieved at 29.0–29.5 °C, with warmer temperatures producing females and cooler temperatures producing males [23, 24]. In *Alligator mississippiensis*, temperatures of 30 °C or lower produce females and temperatures of 34 °C and higher produce males [25].

2 Materials

Working with embryonic material, whether in tissue culture or in situ hybridization, requires a clean working environment. All benches and equipment should be washed and sterilized, and DNase- and RNase-free tips and tubes should be used (*see* **Note 2**). Water should be purified using a Milli-Q system and autoclaved. In some cases (indicated below), water and solutions should be treated for RNases (*see* **Note 3**).

2.1 Harvesting Embryos

1. For in situ hybridization or microcomputed tomography, 1× PBS, pH 7.0–7.4: 130 mM NaCl, 7 mM sodium phosphate dibasic, 3 mM sodium phosphate monobasic, 2 mM EGTA. Dissolve 7.597 g NaCl, 0.994 g Na_2HPO_4, 0.360 g NaH_2PO_4, and 0.9366 g EGTA stepwise into 1 L Milli-Q-purified water. pH to 7.0–7.4 with HCl. Autoclave.
2. For electroporation and ex ovo culture, Howard Ringer's Solution with gentamycin (HR + gent): 0.12 M NaCl, 1.5 mM $CaCl_2$, 5 mM KCl, 50 μg/mL gentamycin. Dissolve 7.2 g NaCl, 0.17 g $CaCl_2$ (anhydrous) or 0.23 g $CaCl_2 \cdot 2H_2O$, and 0.37 g KCl stepwise into 1 L Milli-Q water. Autoclave. Add 50 μg/mL gentamycin to solution before use.
3. 3 mL Syringe with 25 G × 5/8 in. needle or a similarly sized needle.
4. Two sets of forceps (Dumont #5).
5. Fine (iris) scissors.
6. Moria embryo spoon, small or large size.
7. Two 1 mL syringes with a needle size near 26 G. Actual needle size is according to user preference.

2.2 Electroporation

1. 60 mm Petri dish.
2. Dental wax.
3. Pulled borosilicate glass tube with filament (outer diameter (OD) 1.00 mm, inner diameter (ID) 0.50 mm, length 10 cm). Pulled with a flaming micropipette puller to make a finely tipped glass needle.

4. 5 μL of plasmid DNA (5 μg/μL) mixed with a few particles of Fast Green to enable visualization of plasmid DNA solution.
5. Manual micromanipulator connected to a picospritzer.

2.3 Explant Culture

1. 10% Bleach/dH_2O.
2. 6-Well 24 mm Transwell with 3 μm pore polyester membrane insert, sterile.
3. Culture medium: 10% Fetal bovine serum (FBS), 1% glutamine, 50 μg/mL gentamycin, 2.5 μg/mL fungizone, 67 U/mL nystatin, Dulbecco's modified Eagle medium (DMEM) to final volume. If your cultures will examine bone development, add 100 μg/mL ascorbic acid.
4. CO_2 incubator: 33 °C, 5% CO_2.
5. Optional: Drugs or proteins and appropriate solvents to add to culture medium.
6. Optional: Affi-gel blue beads or heparin agarose beads and recombinant proteins for local manipulations.

2.4 Embryo Fixation and In Situ Hybridization

1. The size of your embryos/tissues determines the volumes needed for fixation and processing. Small embryos and explants can be fixed in 1.5 mL Eppendorf tubes, whereas older embryos should be fixed in scintillation vials, 15 mL or 50 mL Falcon tubes.
2. 4% Paraformaldehyde (PFA): Dissolve 4 g paraformaldehyde in 100 mL 1× PBS at 60 °C in a beaker on a magnetic plate with a stir bar. Adjust to pH 7 with NaOH (*see* **Note 4**).
3. 1× PBST: 1× PBS, pH 7.0–7.4 (Subheading 2.1), 0.1% Tween-20. Add 1 mL Tween-20 to 1 L 1× PBS using a syringe (without a needle).
4. Methanol (MeOH): Bottles of 25% MeOH/PBST, 50% MeOH/PBST, 75% MeOH/dH_2O, and 100% MeOH can be prepared for convenience in dehydration and rehydration steps.
5. In situ hybridization can be carried out in sterile multi-well cell culture dishes, with wells that allow proper washing of specimens during steps.
6. Proteinase K (PK): 1 μg/mL in 50 mM Tris pH 8.5, 5 mM EDTA. Add 5 mL 1 M Tris pH 8.5, 1 mL 0.5 M EDTA, and 10 μL of 10 mg/mL stock solution of proteinase K, and fill to 50 mL with Milli-Q. Proteinase K can also be diluted in 1× PBST.
7. 0.2% Glycine in PBST: Add 0.2 g to 100 mL 1× PBST.
8. 0.1 M Triethanolamine (TEA), pH 7–8: Dissolve 18.57 g in 1 L Milli-Q water, and autoclave.
9. 50× Denhardt's solution: 1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) bovine serum albumin fraction V (BSA). Dissolve 1 g Ficoll 400, 1 g PVP, and 1 g BSA

in DEPC water to a final volume of 100 mL. Filter sterilize, and store at −20 °C.

10. 20× Sodium citrate buffer (SSC), pH 7: 3 M NaCl, 300 mM trisodium citrate. Dissolve 175.3 g NaCl and 88.2 g $Na_3C_6H_5O_7$ in 800 mL Milli-Q water. Adjust pH to 7 with glacial acetic acid. Adjust volume to 1 L and autoclave.
11. Hybridization buffer: 50% Formamide, 5× SSC pH 7.0, 1× Denhardt's, 0.1% Tween-20, 10 μg/mL Baker's yeast tRNA, 50 μg/mL heparin. For 100 mL, add 50 mL formamide, 25 mL of 20× SSC pH 7.0, 2 mL of 50× Denhardt's, 1 mL of 10% stock, 100 μL of 10 mg/mL tRNA, and 100 μL of 50 mg/mL heparin, and fill to a final volume with Milli-Q or DEPC water. This buffer is also used in prehybridization (*see* **Note 5**).
12. Digoxigenin (DIG) RNA probe: This should be added to hybridization buffer at a concentration of 0.5–0.75 μg/mL.
13. RNase A buffer: 10 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.1% Tween-20. Combine 1 mL of 1 M Tris–HCl pH 7.5, 10 mL of 5 M NaCl, and 1 mL of 10% Tween-20 to a final volume of 100 mL with Milli-Q water.
14. 50% Formamide, 5× SSC, 0.1% Tween-20: Combine 125 mL formamide, 62.5 mL 20× SSC, and 2.5 mL 10% Tween-20 and add Milli-Q water to a final volume of 250 mL.
15. 1× Maleic acid buffer (MAB): 100 mM Maleic acid, 150 mM NaCl, 0.1% Tween-20. Dissolve 11.61 g maleic acid and 8.766 g NaCl stepwise into 800 mL Milli-Q water. Dissolve 7.8–7.9 g NaOH into solution to bring pH to 7.5. Adjust final volume to 1 L with Milli-Q water. Does not need to be autoclaved, but many users do.
16. 10% Boehringer-Mannheim Block (BMB): Dissolve 10 g Boehringer-Mannheim Blocking Reagent in 1× MAB to a final volume of 100 mL. Store at −20 °C.
17. 1× MABT: 0.1% Tween in MAB. Add 1 mL Tween to 1 L of 1× MAB with a syringe (without a needle).
18. 1% BMB/1× MABT: Add 17 mL of 10% BMB + 153 mL 1× MABT.
19. Anti-digoxigenin-AP, Fab Fragments.
20. 1% BMB/1× MABT +1/5000 anti-DIG antibody: Take 50 mL of 1% BMB/1× MABT and add 10 μL anti-digoxigenin antibody. The antibody should be pre-absorbed in this solution for 1 h at 4 °C prior to use.
21. AP Buffer: 100 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM $MgCl_2$, 5 mM levamisole. Combine 10 mL of 1 M Tris-HCl pH 8.5, 2 mL of 5 M NaCl, 2.5 mL of 2 M $MgCl_2$, and 0.120 g

levamisole in dH_2O water to a final volume of 100 mL. The pH of this solution should be 8.5–9 for the reaction to work. If specimens from alligator are used, add 10% high-molecular-weight polyvinyl alcohol (PVA; weight 40–100 kDa) to the AP buffer (*see* **Note 6**).

22. BM Purple.
23. 50% Glycerol/PBS.
24. Hybridization ovens or water baths, in some cases a PCR machine is useful (*see* **Note 7**).
25. Petri dishes filled with 1% agarose for imaging: In the solidified agarose, the user can dig trenches to hold the specimens for imaging.

2.5 Microcomputed Tomography

1. Bruker SkyScan 1272 high-resolution μCT scanner.
2. Phosphotungstic acid (PTA): Stock solution is 1% (w/v) phosphotungstic acid in water. Working solution is 0.3% PTA in 70% ethanol (*see* **Note 8**).
3. Low-melting-point (LMP) agarose: If samples are large enough to not move within the tube used for scanning, they may be scanned in 70% ethanol. If, however, samples move while being scanned, samples may be immobilized by scanning in 1% LMP agarose (w/v) in 1× PBS.

3 Methods

3.1 Harvesting Turtle and Alligator Embryos

1. If embryos will be used for electroporation or ex ovo culture, begin by rinsing eggs with dH_2O, and then soak for 5 min in 10% bleach/dH_2O, 5 min in sterile dH_2O, and 3–5 min in 70% ethanol. Transfer egg to the ring of modeling clay or other support and air-dry. Dissect embryos in HR + gent rather than PBS. If embryos are harvested for in situ hybridization, spray eggs with 70% EtOH and remove particulate matter from vermiculite (*see* **Note 9**).
2. Candle eggs to determine the position of the embryo along the eggshell (Fig. 1).
3. If desired, puncture the egg using a needle and syringe (or example, 3 mL syringe with 25 G × 5/8 in. needle for turtles, 5 mL syringe with 22 G × 1.5 in. needle for alligators) to suction some thin albumin and alleviate pressure within the egg.
4. For early-stage embryos, cut and remove the eggshell around the embryo with fine scissors, and place this into a petri dish filled with sterile saline (HR + gent or 1× PBS). Carefully separate the embryo from the eggshell and transfer to a new

dish filled with saline. For older embryos, cut a window into the eggshell (Fig. 1), remove the embryo with a moria embryo spoon, and place it into a dish with saline. Smaller embryos can be transferred with sterile plastic pipettes.

5. Dissect away extraembryonic membranes with forceps and/or needles on syringes.

3.2 Electroporation

1. Incubate turtle eggs until desired stage. For trunk neural crest cell transfection, eggs are incubated to G10 and embryos are isolated as described in Subheading 3.1.
2. Prepare a 60 mm petri dish with two parallel strips of dental wax secured to the bottom of the dish. Place the strips close together (approximately 3–5 mm apart) and remold with forceps to create a depression for embryos to lay ventral side down (Fig. 2a). The width of the strips will depend on the stage of the embryo. When the strips are in place, fill the dish with sterile 1× HR + gent.
3. Place the embryo into the depression between the dental wax strips, dorsal side up (Fig. 2a).
4. Insert the electrodes into the cables attached to a BTX electroporator (positive is red, negative is black; Gemini twin-wave electroporation system, 45-2040 or ECM 830 square-wave electroporation system, 45-0002, BTX). Electrodes can either be commercially produced (Genetrodes, BTX) or made in the lab. In this application, we used handmade platinum electrodes (based on [26]).
5. For neural crest injection and electroporation delivery, set the electroporator to
   (a) Voltage = 50 V
   (b) Pulse length = 45 ms
   (c) Pulses = 5
   (d) Interval = 1 s (*see* **Note 10**)
6. Load the solution of 5 μL plasmid DNA + Fast Green particles into the pulled glass needle.
7. Insert loaded pulled glass needle into the needle holder of a micromanipulator connected to a picospritzer.
8. Gently break a small part of the pulled glass needle tip off with forceps and carefully discard to glass waste. Make sure that a small volume of liquid comes out of the glass needle when the picospritzer is activated (*see* **Note 11**).
9. Set the picospritzer to
   (a) Pressure = 20 psi
   (b) Duration = 100 ms

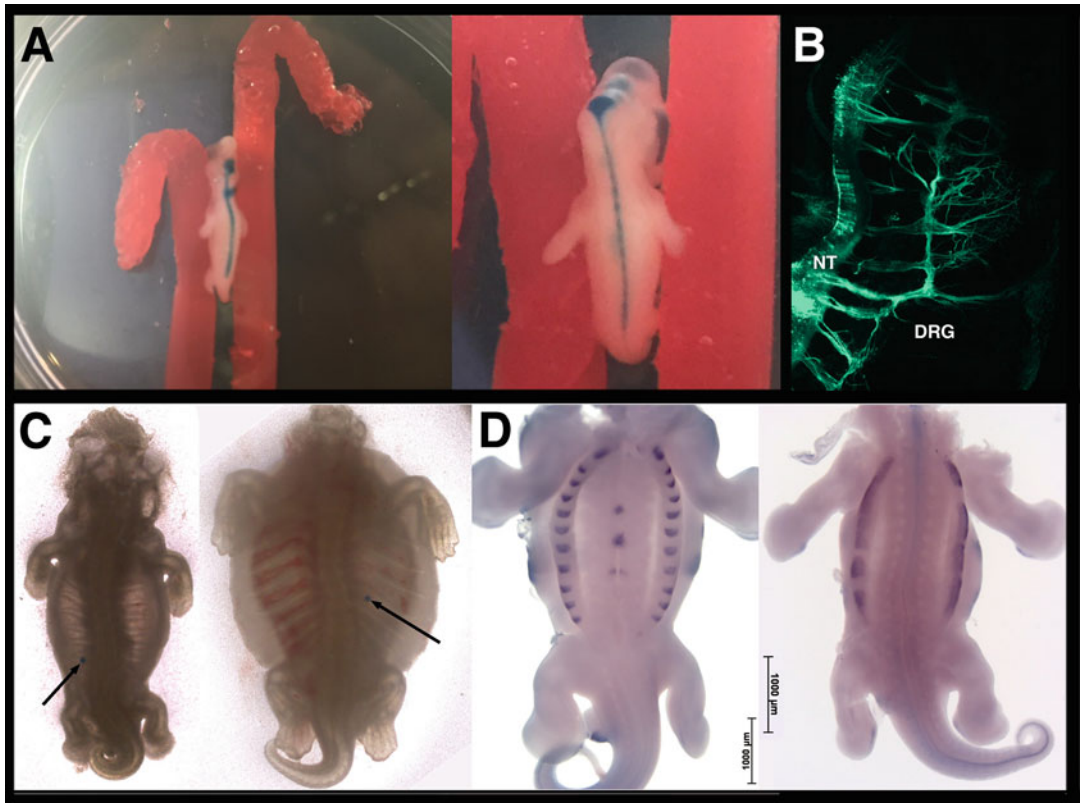

Fig. 2 Ex ovo manipulations using turtle embryos. (**a**) Parallel strips of dental wax in petri dish with embryo positioned between wax, dorsal side up for injection into the neural tube. The addition of Fast Green to the plasmid DNA aids in the visualization of the injection. (**b**) Stacked confocal image showing neural crest cell derivatives (dorsal root ganglia, DRG) labeled by successful electroporation of a YFP-expression vector into the neural tube (NT) of a turtle embryo. (**c**) Decapitated, eviscerated turtle embryonic explants on Transwell filters after culture for 4 days. Using this method, proteins or drugs may be added directly to the media or to beads (blue; black arrows). (**d**) Results of culture experiments with SU5402 added to the media assayed by the expression of placodal signaling center marker *Shh* (purple). In the left control embryo, the pattern is as expected with 12 separated scute primordia on each side of the developing turtle shell. In the right SU5402-treated embryo, the pattern of signaling centers has changed. Anterior is to the top in all images

(c) and adjust as needed (the pressure and duration depend on the size of the tip of the glass needle, thickness of the liquid to inject, and size of the subregion to be labeled).

10. Position the picospritzer to inject plasmid DNA into the neural tube lumen of the embryo held in the dental wax.
11. Inject the plasmid DNA into the site of interest and retract the glass needle.
12. Place the electrodes into the HR + gent solution, on either side of the embryo, parallel and adjacent to the region of the embryo that needs to be transfected (positive on the right and negative on the left).

13. Activate the electroporator and hold the electrodes steady until the pulses have stopped.
14. Remove the embryo from the dental wax mold and prepare for culturing as described in Subheading 3.3.
15. Following culturing, embryos are best imaged using confocal microscopy (Fig. 2b). Fluorescence can be detected 12 h following electroporation.

3.3 Explant Culture

1. Following isolation, transfer 2–3 embryo groups to a fresh dish with HR + gent to minimize cross-contamination.
2. Remove head and open embryo along ventral midline through pelvis using fine forceps. Remove viscera including heart, digestive tract, and mesonephros on either side of aorta.
3. Transfer desired number of Transwell Clear filters to 6-well plate. Add 1.8 mL of explant medium under the filter. It is helpful to have additional medium in empty wells.
4. Add 1–2 drops of explant medium to filter and place embryo into the drop. Gently spread out until ventral surface is in contact with filter (Fig. 2c). Place 2–3 explants on each filter and culture at 33 °C and 5% CO_2. Explants can be cultured for up to 3 weeks, and media should be changed every 2 days.
5. Drugs or proteins can be added to explant culture medium to examine their effects on development. When adding these to the culture medium, be sure to add the solvent that does not contain the drug or protein (for example, DMSO or BSA) to the control cultures.
6. To test for local effects of proteins or inhibitors on the explant, protein-soaked beads can be added to explants. Heparin agarose beads or more general binding Affi-gel blue beads should be washed with PBS, and then soaked for 1 h at 37 °C in recombinant proteins or BSA (control). Beads are added to the region of interest, and explants are cultured normally (Fig. 2c). Results may be assayed by in situ hybridization (Fig. 2d; Subheading 3.4) and/or microcomputed tomography (Fig. 3; Subheading 3.5).

3.4 Embryo Fixation and In Situ Hybridization

1. Following dissection in 1× PBS or HR + gent, fix specimens in 4% PFA (*see* **Note 12**).
2. Following fixation, wash specimens three times in 1× PBS. For small specimens, washes can be 5 min each. For larger specimens, washes should be 15–30 min each (*see* **Note 13**).
3. Dehydrate specimens stepwise in 25% MeOH/PBST, 50% MeOH/PBST, 75% MeOH/PBST, and then 2× in 100% MeOH. Specimens are stored in 100% methanol at −20 °C until they undergo in situ hybridization.

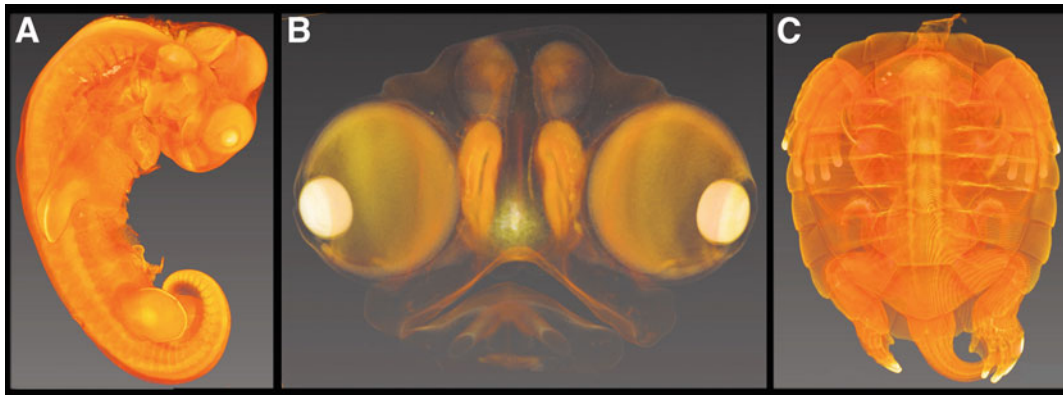

Fig. 3 Morphological assessment by microcomputed tomography (microCT). (**a**) A young turtle embryo showing dense staining in the developing limb buds. (**b**) This method was used to study craniofacial morphogenesis with a special focus on the oral epithelium [27]. (**c**) An older turtle embryo used to visualize the pattern of scute formation in the turtle shell and the relation of scutes to the developing ribs [5]

4. Divide specimens into wells of sterile trays filled with 100% methanol so that there is a sufficient volume of liquid washing the specimen(s). Specimens may be grouped into the same well if the same probe will be used.
5. Steps are carried out with rocking and at room temperature unless indicated. The hybridization oven can be turned on to 60 °C and a heat block to 80 °C.
6. Rehydrate: wash for 10 min each in 75% MeOH/PBST, 50% MeOH/PBST, and 25% MeOH/PBST.
7. Wash embryos three times with 1× PBST.
8. Treat with proteinase K (10 mg/mL) for 5–12 min, depending on the size of the embryo (5 min for cultures from Subheading 3.3, 10–12 min for most other stages).
9. Wash with 2 mg/mL glycine in PBST for 10 min to stop the proteinase K reaction.
10. Rinse two times for 5 min in 0.1 M TEA.
11. Wash in 0.1 M TEA +2.5 μL/mL acetic anhydride for 5 min.
12. Wash in 0.1 M TEA +5 μL/mL acetic anhydride for 5 min.
13. Wash two times for 5 min in 1× PBST.
14. Refix for 20 min in 4% PFA.
15. Wash embryos three times with PBST.
16. Rinse with a 1:1 solution of PBS:hybridization buffer for 10 min.
17. Prehybridize with "prehybridization" buffer (same as hybridization buffer) for ≥1 h at 60 °C. You will save this buffer in trays under the hood for reuse the following day.

18. Denature probes in hybridization buffer (0.5–0.75 μg/mL) at 90 °C for 3 min (close lids of Eppendorf tubes with locks), and then transfer to ice. Distribute 500 μL per well to 24-well trays and transfer specimens (alternatively, one could use 5 mL conical tubes or larger welled trays if specimens are large; be sure to cover specimen completely with hybridization buffer + probe). Wrap in plastic wrap and foil, and hybridize overnight at 60 °C with probe (*see* **Note 7**).
19. Transfer embryos back to trays with saved prehybridization buffer and wash for 10 min at 60 °C. Turn on another oven or water bath to 37 °C.
20. Wash two times for 30 min each with hybridization buffer at 60 °C.
21. Let embryos cool to room temperature.
22. Rinse in a 1:1 solution of hybridization buffer:RNase A buffer at room temperature for 5 min.
23. Wash with RNase A buffer for 5 min.
24. Treat with RNase A (100 μg/mL) in RNase A buffer for 30 min at 37 °C.
25. Wash with a 1:1 solution of RNase A buffer:SSC/formamide/Tween-20 to 60 °C.
26. Wash with SSC/formamide/Tween-20 at 60 °C for 5× 30 min to 1 h.
27. Wash with a 1:1 solution of SSC/formamide/Tween-20:PBST for 10 min at room temperature.
28. Wash with 1× PBST two times for 10 min.
29. Wash with 1× MABT two times for 10 min.
30. Block for ≥1 h with 1% BMB/1× MABT at 4 °C. Pre-absorb the antibody at 4 °C during this time.
31. Incubate with 1% BMB/1× MABT + 1/5000 anti-DIG Ab overnight at 4 °C.
32. Wash three times for 5 min in 1× PBST at 4 °C.
33. Wash four times for 1 h in 1× PBST at 4 °C.
34. Wash 1× 10 min, and 1× 20 min in AP buffer at room temperature. This raises the pH of the tissue for the color reaction.
35. Incubate in BM Purple at room temperature or overnight at 4 °C. Samples should be kept in the dark during this step, for example, by covering the tray with foil. The time for the color reaction to develop is probe and tissue dependent (Fig. 2b), and could range from several hours to several days. Incubation at 37 °C speeds up the reaction, but can also result in higher background staining.

36. Wash three times in 1× PBS to stop the reaction.
37. Fix in 4% PFA overnight at 4 °C or for 2 h at room temperature.
38. Rinse two times for 10 min in 1× PBS.
39. Gradually transfer to 25% glycerol/PBS, and then 50% glycerol/PBS. This helps to clear the specimens.
40. Image specimens in 1× PBS on agarose-filled petri dishes.

3.5 Microcomputed Tomography (microCT) of Embryonic Turtles

MicroCT gives a 3-dimensional perspective on the morphology of interest, but does not require fluorescent signals as in confocal microscopy. Rather, contrast can be achieved by the use of stains that react with the extracellular matrix of the tissue ([15]; Fig. 3). We first used this to visualize the developing placodes that give rise to turtle scutes, in relation to the developing shell as a whole. Because we were not able to easily visualize scute patterns from cultured specimens using in situ hybridization or conventional microscopy, we used microCT to show this pattern [5]. A very nice comparison that can be done is in situ hybridization followed by microCT on the same specimen to compare deeper histology 3-dimensionally. The color precipitate from the in situ is not stable in alcohol and will disappear during staining for microCT; therefore, all imaging of the in situ experiment must be done beforehand.

1. Following fixation in 4% PFA (or following imaging of in situ), wash 2–3 times in 1× PBS, and gradually dehydrate specimen to 25% ethanol/PBS, 50% ethanol/PBS, and finally 70% ethanol/dH_2O.
2. Stain in 0.3% PTA/70% ethanol overnight at room temperature. For very large, differentiated specimens, this may take 2–3 days or even 1 week.
3. Wash 2–3 times in 70% ethanol (10 min each for smaller specimens, 30 min each for larger specimens). Store in 70% ethanol for microCT.
4. Scan specimen in 70% ethanol. Use smallest vessel possible (0.2 μL PCR tubes, 1.5 mL Eppendorf tubes, 15 mL conical vials), for scanning. Because the tube and specimen rotate during the scanning, the specimen must be immobilized (*see* **Note 14**).

4 Notes

1. If turtle eggs are transported in hypoxic conditions, the vermiculite should be supplemented with a greater amount of water than normal when first incubating the eggs.

2. Conditions for working with RNA and organ cultures are demanding, and all working areas should be cleaned with RNase AWAY, bleach, and/or 70% ethanol when appropriate. Tools can be soaked in 0.1 N NaOH and 1 mM EDTA for 15 min and then rinsed extensively with clean (Millipore-filtered or DEPC-treated) water. Gloves should be worn for RNA work. For organ culture, wash hands and tools with soap, and spray tools with 70% ethanol before use. If possible, sterilize tools with a dry bead sterilizer. Needles (Becton-Dickinson, for example) come sterilized and are a convenient source of sharp tools; these should be discarded after each use. If it is not possible to dissect tissues for culture under a sterile hood, then one should wear gloves. However, care must be taken, as bottles of media and solutions should be flamed when opening and closing.
3. In clean facilities (laboratories that are cleaned for RNA work or organ culture), most solutions can be made with ultrapurified water (Milli-Q-sterilized) and autoclaved. If there is any concern about RNase contamination, the user should consider diethyl pyrocarbonate (DEPC)-treating water and solutions where appropriate. DEPC-treated water can be purchased, but it can also be made easily: add 1 mL DEPC to 1 L water, shake vigorously to bring DEPC into solution, incubate solution overnight (12 h) at 37 °C, and autoclave. DEPC-treated water should always be used in RNA probe synthesis.
4. Paraformaldehyde solution should be stored at −20 °C until use. If you plan to use large quantities of PFA, you may make 20% PFA stock solutions (20 g of paraformaldehyde in 1× PBS), store in freezer, and then dilute to 4% PFA when needed. 4% PFA will also be used during the in situ.
5. Larger volumes of hybridization buffer can be made and stored in −20 °C.
6. Polyvinyl alcohol has been reported to increase sensitivity of whole-mount in situ hybridizations. The color reaction at the end of the in situ hybridization with alligator (crocodilian) tissue should have 10% polyvinyl alcohol added to the AP buffer to enhance the reaction without increasing background. We have not found this to be necessary for turtle tissues; however, if background is a problem with other organisms, addition of this reagent is recommended.
7. Hybridization ovens or water baths may be used for the in situ hybridization. When using water baths, make sure to wrap trays in plastic wrap twice, followed by aluminum foil twice to prevent water from entering the trays. Hybridization temperature should be determined empirically for each probe. Generally, 60–70 °C works well for most probes. Because testing multiple

temperatures can be laborious, we have successfully used gradient PCR with small samples (early embryos, small limbs, alligator tails, for example) to optimize the hybridization temperature. Samples should be fully submerged in 100 μL of hybridization buffer in 0.2 mL PCR tubes. Program the PCR machine to first denature the probes at 90 °C for 3 minutes, then bring the solution to an annealing temperature (or range if using gradient PCR) forever for hybridization. Prehybridization may be carried out at 60 °C, then transfer embryos to PCR tubes once the hybridization temperature is stable. Posthybridization washes may be done at 60 °C.

8. The PTA stain used in microCT is permanent. Iodine-based stains [15] are reversible.
9. If embryos are harvested for in situ hybridization, antibody staining, or microcomputed tomography, then dissection should be done in 1× PBS. If embryos will be used for ex ovo culture, then Howard Ringer's Solution with gentamycin should be used instead to minimize the risk of contamination. Fungal and bacterial contamination is a big issue with turtle embryo cultures. To minimize this, the eggs should be cleaned thoroughly and the embryos should be separated into small groups during isolation.
10. Settings on electroporator (especially voltage), as well as placement of the electrodes, will have to be adjusted depending on the age/size of tissue and transfection target. Our recommended settings have been optimized for neural crest injection in stage G10 turtle embryos. Efficacy is determined by the expression of reporter vectors.
11. Only a small amount of liquid should come out of the needle to target cells or tissues of interest, and the volume should not spread beyond the target tissue. If too much is coming out after breaking the tip, lower the picospritzer duration or pressure so that only a small ball of liquid forms on the end of the tip. If the tip end is too blunt, it will not pierce through the embryonic tissue cleanly. When this occurs, discard needle and make a new one.
12. Use a large volume of fixative; about ten times the volume of the specimen is preferable. It is important to rock or rotate the specimens during fixation. Specimens can be fixed for 2 h at room temperature or overnight at 4 °C. Museum specimens are generally fixed in 10% buffered formalin. Though this may be sufficient for immunohistochemistry and microCT, in situ hybridization requires RNase-free processing and storage. Therefore, 4% PFA is recommended for whole-mount in situ.

    Larger specimens should be fixed at least overnight, and if one is only interested in a certain region or organ system, the user is

advised to dissect the tissue as small as possible. Depending on the tissue or organ of interest, the user may prefer to perform in situ hybridization on sectioned tissue. If the specimen is so large that the experiments should be done on sections, then use 4% PFA for fixation, vibratome section (30–200 μm) in low-melting-point agarose, and process sections as in the whole-mount protocol. If thin sections are desired (7–10 μm), it is recommended to fix in "formoy" (6 parts 100% ethanol, 3 parts 37% formaldehyde, 1 part glacial acetic acid), store in 100% ethanol, and section in paraffin wax.

13. The volumes in materials are suggested amounts that the user should adjust depending on the volume of their experiment. For the in situ, specimens should be completely covered by liquids with sufficient volume for washing.
14. A good trick for this is either to add cotton to the tube with the specimen, which will also make separation of multiple specimens easier, or to scan in LMP agarose rather than ethanol. It is advisable to scan experimental samples at the same time to minimize variation between scans.
15. *General note*: These methods have been used on various organisms and tissues [27, 28] , and will likely work on other reptiles. Of note, however, is that some tissues are far more difficult to penetrate than others and may take optimization.

Acknowledgments

This work was supported by the Academy of Finland (JEM-V), Stowers Institute for Medical Research (RM, PMK), and NSF grant IOS-145177 (JACT). We are grateful to Kevin Padian, Richard Harland, Marvalee Wake, Scott Gilbert, Concordia Turtle Farm, Kliebert's Turtle & Alligator Farm, and Ruth M. Elsey and the Louisiana Department of Wildlife and Fisheries.

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Chapter 15

Methods for Isolating the Balbiani Body/Germplasm from *Xenopus laevis* Oocytes

Amanda Butler, Dawn Owens, Mary Lou King, and Tristan Aguero

Abstract

The Balbiani body (Bb) is a large membrane-less organelle, densely packed with mitochondria, endoplasmic reticulum, proteins, and RNA. The Bb is present in many vertebrate female gametes. In frogs, the Bb is established early during oogenesis and operates as a maternal inherited embryonic determinant that specifies germline identity through the formation of germplasm. We describe here two techniques to isolate the Bb/germplasm from *Xenopus laevis* primary oocytes.

Key words Balbiani body-mitochondria cloud, Germplasm, Germ cells, Oogenesis, *Xenopus*

1 Introduction

The Balbiani body (Bb), also known as mitochondrial cloud, is a transitory and complex assembly of mitochondria, smooth endoplasmic reticulum, Golgi cisternae, maternal proteins, and RNA. In both mice and frogs, the Bb develops adjacent to the nucleus at the onset of oogenesis [1–4]. In *Xenopus*, the Bb acts as a carrier to transport germplasm components to the vegetal pole of the oocyte, positioning it within the protective environment of the future endoderm [5, 6]. The germplasm constitutes a special part of oocyte cytoplasm, formed by maternally inherited proteins and RNAs required for the specification and maintenance of germline identity [7]. The germplasm is asymmetrically assembled at the tip of the Bb closest to the vegetal pole [8]. Most of the maternal mRNAs present in the Bb correspond to germplasm components. However, some mRNAs are also involved in somatic patterning, such as wnt11 and syntabulin [9–11], which mobilize within the Bb to reach the oocyte vegetal pole. The Bb has also been shown to be involved in the formation of lipid droplets, and the selective elimination of dysfunctional mitochondria during oogenesis [12, 13]. In each oocyte, the successful formation of the germline and healthy

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_15,

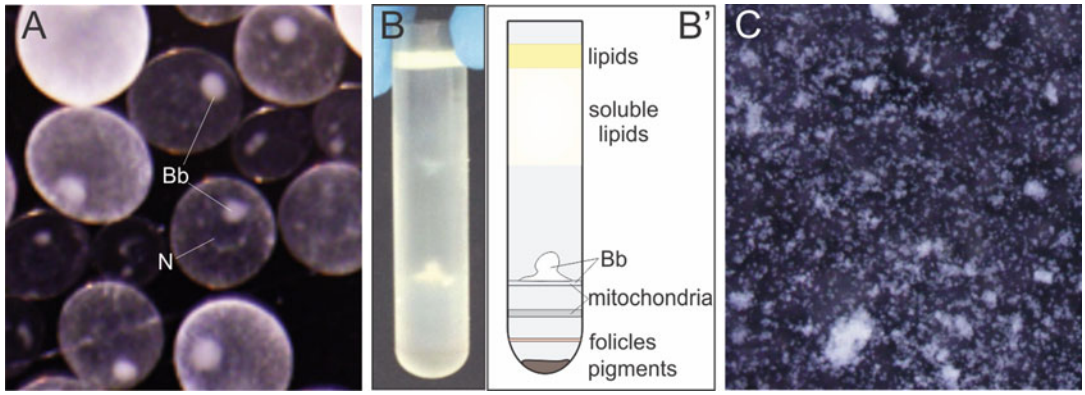

Fig. 1 Gradient isolation of Bb and germplasm. (**a**) Isolated stage I–II Xenopus oocytes with Balbiani bodies (Bb). (**b**, **b′**) Ultracentrifuge tube showing the white ball corresponding to the Bb. A representation of all compounds obtained after centrifugation is shown in **b′**. (**c**) Magnification of Bb fluffy material obtained from Percoll gradient. *N* nucleus

mitochondria is required to pass on critical genetic information to future generations.

Xenopus laevis provides exceptional advantages to study the Bb. Oocytes are both abundant and easily isolated. Their large size allows them to be smoothly manipulated. According to Dumont 1972 [14], *Xenopus laevis* oocytes are classified in six well-defined stages. Previtellogenic stages I and II oocytes contain clearly visible Balbiani bodies, initially in contact with the nucleus or germinal vesicle and, subsequently, directed toward the vegetal cortex (Fig. 1). The Bb is a large and spherical amyloid-like structure with a diameter of 30–40 μm, oriented toward the oocyte's vegetal pole [15, 16] (Figs. 1 and 2). As oocyte development continues, the Bb translocates to the vegetal pole and progressively disperses into germplasm islands at the vegetal cortex of stage VI oocytes [5, 18]. After fertilization, germplasm gradually accumulates into larger yolk-free pools along the vegetal cortex [1], asymmetrically passing into only one daughter cell during the cleavage stages of embryogenesis [19, 20]. In the developing embryo, the descendants of vegetal blastomeres, containing Bb-derived germplasm, become progenitors of the germ cells [21, 22]. The preservation of germline fate is one of the most critical events during embryo development as germ cells are the only cell population that can transmit genetic information to the progeny [1, 5].

2 Materials

Prepare all solutions using deionized sterile water and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated).

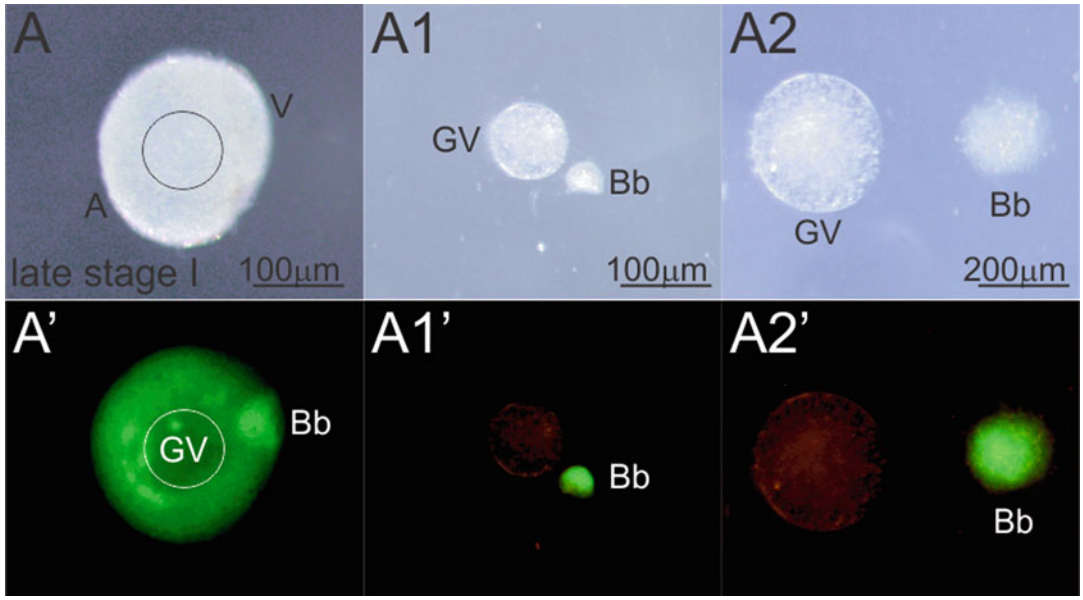

Fig. 2 Manual isolation of Bb. (**a**, **a**′) Bright-field (**a**) and fluorescence (**a**′) images of a late stage I Xenopus oocyte incubated with a low concentration of *DiOC6* (3,3′-dihexyloxacarbocyanine iodide) to highlight the Bb or *mitochondria cloud* [17]. Notice that at this stage the Bb is separated from the GV. (**a**1–**a**2′) Manually isolated GV and Bb. (**a**2, **a**2′) Magnification of **a**1, **a**1′. *A* animal side, *V* vegetal side, *Bb* Balbiani body, *GV* germinal vesicle or nucleus

2.1 Stage I–II Oocyte Isolation

1. *Xenopus laevis* oocytes (defolliculated) (*see* **Note 1**).
2. Collagenase (Type 1; Worthington Biochemical) or Liberase TM (Roche).
3. Dissecting microscope and dual-gooseneck LED illuminators (low heat).
4. Forceps (e.g., Dumont #4 or #5) (*see* **Note 2**).
5. Sieves (Pro 4″ or Low Pro 4″) with 475 and 1000 µm mesh (Aquaculture Nursery Farms).
6. Hair loop.
7. Incubator at 18–20 °C.
8. Petri dishes (35 mm, 100 mm) and/or 6well microplates (35 mm/well) (plastic).
9. Modified Barth's Saline (MBS) for oocytes (1×): 0.7 mM $CaCl_2$, 5 mM HEPES, 88 mM NaCl, 1 mM KCl, 2.5 mM $NaHCO_3$, and 1 mM $MgSO_4$, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Combine 100 mL 10× MBS salt solution, 7 mL 0.1 M $CaCl_2$, and 5 mL penicillin-streptomycin mix (10 mg/mL penicillin [100×] and 10 mg/mL streptomycin [100×] in water). Make up to 1000 mL with deionized water. Store for up to 1–2 months at 16–18 °C (same temperature used for oocyte incubation). Check before use.

10. 10× stock MBS salt solution: 50 mM HEPES, 880 mM NaCl, 10 mM KCl, 25 mM $NaHCO_3$, and 10 mM $MgSO_4 \cdot 7H_2O$. Dissolve 11.92 g HEPES, 51.43 g NaCl, 0.75 g KCl, 2.10 g $NaHCO_3$, and 2.46 g $MgSO_4 \cdot 7H_2O$ in ~800 mL deionized H_2O; adjust the pH to 7.8 with 10 M NaOH; then make up to 1000 mL with deionized water; sterilize by autoclaving; and store at room temperature.
11. 0.1 M $CaCl_2$ (for MBS): Dissolve 1.11 g of $CaCl_2$ in 50 mL of deionized H_2O and then make up to 100 mL with deionized water; store at room temperature indefinitely.
12. *1*× Oocyte reagent 2 *(O-R2)*: 5 mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3.8 mM NaOH, 1 mM Na_2HPO_4, 100 μg/mL penicillin and 100 μg/mL streptomycin. Combine 100 mL of O-R2 10× Stock Solution A, 100 mL of OR-2 10× Stock Solution B, 5 mL penicillin-streptomycin mix (10 mg/mL penicillin [100×] and 10 mg/mL streptomycin [100×] in water) and 795 mL of deionized H_2O. Store for up to 1–2 months at 16–18 °C (the same temperature used for oocyte incubation). Check before use. The pH should be 7.8.
13. O-R2 10× Stock Solution A: 50 mM HEPES, 825 mM NaCl, 25 mM KCl, 10 mM $CaCl_2 \cdot 2H_2O$, 10 mM $MgCl_2 \cdot 6H_2O$, and 38 mM NaOH. Dissolve 11.915 g HEPES, 48.221 g NaCl, 1.864 g KCl, 1.47 g $CaCl_2 \cdot 2H_2O$, 2.03 $MgCl_2 \cdot 6H_2O$, and 1.520 g NaOH in 800 mL of deionized H_2O and then make up to 1000 mL with deionized water. Store indefinitely at 4–8 °C.
14. O-R2 10× Stock Solution B: 10 mM Na_2HPO_4. Dissolve 1.420 g of Na_2HPO_4 in 800 mL of deionized H_2O and then q.s. to 1000 mL with deionized water. Store indefinitely at 4–8 °C.

2.2 Balbiani Body Purification Using a Percoll Gradient

1. Percoll PLUS (GE Healthcare; kept at 4 °C) solutions: 60%, 20%, 10%, and 5% (v/v) Percoll PLUS in TES.
2. Tris-EDTA (TE) solution: 10 mM Tris pH 7.5, 1 mM EDTA (RNase free). TE can be made up beforehand.
3. Tris-sucrose-EDTA (TES) buffer: 0.25 M Sucrose in TE (RNase free). Make fresh before use. Aliquot an appropriate amount and add protease inhibitor cocktail before use. Use 1 complete Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche) per 10 mL of TES buffer. Alternative protease inhibitor cocktails can be purchased from various vendors. Use the dosage specified by the manufacturer.
4. Centrifuge and associated rotor.
5. High-speed ultracentrifuge and associated rotor.
6. Incubator at 18–20 °C.

2.3 Manual Balbiani Body Isolation from Stage I–II Xenopus Oocytes

1. Transition buffer (TB): 10 mM HEPES pH 7.4, 2.5 μM DTT, 7 mM $MgCl_2$, 70 mM NH_4Cl_2. Prepare 50–100 mL. Store for up to 1 month at 4–8 °C.
2. Forceps (e.g., Dumont #4 or #5) (*see* **Note 2**).
3. Capillary tubes (e.g., Drummond Scientific 3-000-203G/X [borosilicate glass, 1.14 mm outer diameter, 0.53 mm inner diameter, 90 mm long]).
4. Pipette puller (e.g., Narishige PC-10 or Sutter Instrument P-97).
5. Dissecting microscope and dual-gooseneck LED illuminators (low heat).
6. Hair loop.
7. Incubator at 18–20 °C.
8. Petri dishes (35 mm, 100 mm) and/or 6-well microplates (35 mm/well) (plastic).

3 Methods

Two methods to isolate the Bb are described. The first one is the traditional isolation method using a Percoll gradient and the second one is the manual isolation of the Bb in a stabilization medium. The election of the appropriate method will depend on the purpose of the experiment. The gradient isolation allows for processing of a large quantity of Balbiani bodies, is faster, and is not technically challenging. However, preliminary analysis shows that this approach recovers less than a fifth of the proteins that were identified using the manual isolation protocol (our unpublished data). Therefore, although manual isolation of Balbiani bodies is more technically challenging and less material is obtained, it allows for more complete recovery of Bb proteins.

3.1 Xenopus Oocyte Isolation

The selection of the oocyte-donor female will depend on the desired stage of oocytes. In Xenopus, oogenesis is divided into six stages, I–VI [14]. Sexually mature females will yield higher numbers of late-stage (V–VI) oocytes, whereas juvenile females may have predominantly early-stage (I–II) oocytes with few, if any, later staged oocytes (*see* **Note 3**).

1. Harvest ovarian tissue by surgery from anesthetized frogs (*see* **Note 4**). After surgical harvest, the ovarian tissue is placed in MBS or OR2, cut into small pieces (~0.5–1 cm), and then teased apart with forceps to expose the oocytes for defolliculation. Examine the oocytes prior to defolliculation to determine the quality and proceed only if the oocytes are healthy (*see* **Note 5**).

2. Carry out enzymatic defolliculation by digesting tissue pieces at room temperature (22–24 °C) with rotation in either 2 mg/mL collagenase type 1 (Worthington, ~0.23 WU/mL) or Liberase TM (Roche) diluted to ~0.26 WU/mL in medium MBS or OR2 (*see* **Notes 6–9**).
3. Isolate stage I (50–300 μm) and II (300–450 μm) oocytes, by passing the freed oocytes over a 475 μm mesh (nylon or polypropylene mesh) [23]. The oocytes that pass through the mesh (these will be primarily stage I–II oocytes) are collected, placed in fresh OR2, and kept at 16 °C–18 °C (*see* **Notes 10** and **11**).

3.2 Balbiani Body Purification Using a Percoll Gradient

1. Remove stage I–II oocytes from at least four juvenile frogs (*see* **Note12**).
2. While ovary pieces are undergoing enzymatic defolliculation (to free oocytes) in Liberase, make up the Percoll gradients—60, 20, 10, and 5% (v/v) Percoll PLUS in TES (make TES fresh before use). Incubate oocytes in O-R2 media plus antibiotics until use.
3. 2.5 mL of each of the four Percoll layers is needed, so if two samples are being processed (depending on the size of oocyte "pellet"), make up 10 mL of each gradient in 15 mL conical tubes, cap, and put at 4 °C until ready to use.
4. Make Percoll gradient in 14 × 89 mm Beckman centrifuge tubes by slowly adding 2.5 mL of each 60, 20, 10, and 5% Percoll solutions; put the tubes upright in a rack; cover with parafilm; and put them at 4 °C until ready to use.
5. Transfer the isolated stage I–II oocytes to a sterile 1.5 mL (or 2 mL depending on how many there are) microfuge tube.
6. Let the oocytes settle and estimate the volume of oocytes present (based on the markings on the tube). As the oocytes are settling, make up TES solution + protease inhibitors (*see* **Note 13**).
7. Remove as much buffer as possible without disturbing oocytes and add the same volume of TES + protease inhibitors as there are oocytes (estimated in **step 6**) (*see* **Note 14**).
8. Use a 1 mL sterile syringe and needle to lyse the oocytes, and pass them through the needle seven times (*see* **Note 15**).
9. Layer the oocyte lysate very carefully onto the Percoll gradient (approximately 1 mL per tube), and then centrifuge for 45 min at 107,000 × *g* and 4 °C in a SW41 Ti rotor.
10. After centrifugation, a grayish band corresponding to mitochondria will be visible and on top of that a grayish/white ball of fluffy material with a "wisp" (Fig. 1)—this is the Balbiani body aggregate. Collect the Bb using a 200 μL pipettor and transfer to a fresh, sterile 1.5 mL microfuge tube (*see* **Note 16**).

11. It is possible to either freeze the Bb sample at this point at −20 °C and process at a later date or continue with RNA or protein isolation following the appropriate protocol.

3.3 Manual Balbiani Body Isolation From Stage I–II Xenopus Oocytes

1. Isolate stage I–II oocytes from juvenile female frogs using Liberase digestion.
2. Collect stage I–II oocytes in a clean 100 mm glass petri dish containing O-R2.
3. Transfer ~200–300 stage I–II oocytes for processing into a sterile 1.5 mL tube (*see* **Note 17**).
4. After transferring the oocytes that will be processed, cover the petri dish containing the remaining oocytes, and store them at 18–19 °C.
5. Let oocytes for processing settle in the tube, then remove the excess O-R2 (without disturbing the oocytes), and add ~1 mL of ice-cold transition buffer (TB).
6. Let oocytes settle, remove TB (without disturbing the oocytes), and add a fresh ~1 mL of ice-cold TB.
7. Incubate oocytes in TB at room temperature for ~10–15 min (oocytes can remain in TB for up to 45 min).
8. Transfer ~50–100 of settled oocytes in TB into a clean 35 mm glass petri dish containing O-R2.
9. Begin manual isolation of Bbs (it is important to previously work out a technique that will be successful in each user hand). We isolate Bbs using two sharp forceps to peel away the cell membrane leaving the Bb attached to the nucleus or germinal vesicle (GV). We then use a pair of forceps to hold the GV as close to the Bb as possible and use the other forceps to carefully cut the Bb free. Alternatively, a pulled glass needle (similar to what is used for oocyte/embryo microinjections) can replace one pair of forceps to detach the Bb from the GV (*see* **Note 18**).
10. Collect the isolated Bbs (in as little volume as possible) using a P10 pipette and transfer them to a sterile 1.5 mL tube containing ~0.5 mL of OR2 on ice (*see* **Note 19**).
11. Repeat until ~30–50 Bbs are collected.
12. Spin down the Bbs at 12,000 × *g* in a tabletop centrifuge for 10 min at 4 °C, and then put the tube back on ice. Pool additional Bbs in the same microfuge, by repeating the process with recentrifugation of the tube before adding each additional 30–50 Bbs (*see* **Note 20**).
13. Periodically remove and discard the remaining oocytes and debris and add fresh O-R2 to the glass dish.

14. Transfer another ~50–100 oocytes from the TB into the dish containing O-R2 and continue with the procedure.
15. Repeat **steps 8–13** until all the oocytes in the TB have been used or until the oocytes have been in the TB for ~45 min.
16. Once all oocytes in O-R2 have been used, begin at **step 3** with a fresh, sterile 1.5 mL tube and a new batch of 200–300 stage I and II oocytes (*see* **Note 21**).
17. Perform a final spin at 12,000 × *g* in a tabletop centrifuge for 30 min at 4 °C (*see* **Note 22**).
18. Remove supernatant and collect it into a new tube to be analyzed for lost protein (optional).
19. Freeze and store the Bb pellet and supernatant at -80C until analysis (of RNA or protein) (*see* **Note 23**).

As a reference, a protocol for germplasm isolation from vegetal cortex in stage VI oocytes can be found in Owens et al., 2017 [24], and a protocol for primordial germ cell (PGC) isolation from gastrula/neurula Xenopus embryos can be found in Butler et al., 2017 [25].

4 Notes

1. Oocytes should be obtained surgically from anesthetized adult females or sourced from commercial vendors (e.g., Nasco, EcoCyte Bioscience, Xenoocyte) and defolliculated.
2. Two pairs of forceps are used: either two sharpened or one sharpened and one shaped like blunt pliers, according to preference.
3. It is convenient to use late stage I or stage II oocytes rather than early stage I because by late stage I the Bb is detached from the germinal vesicle, and therefore allows for easier isolation of the Bb.
4. Surgeries and survival surgeries must follow specific Institutional Animal Care and Use procedures. Contact the IACU Committee (IACUC) at your institution for exact guidelines.
5. Healthy stage I oocytes will have a white, compact mitochondrial cloud; stage II oocytes will have a disk/cone of mitochondrial cloud material attached to the vegetal cortex. Unhealthy stage I and II oocytes will have diffuse mitochondrial clouds and/or a deflated appearance.
6. Calcium is required for optimal collagenase activity and is included in the medium. Approximately 10 mL of ovarian tissue pieces can be digested in 25 mL of enzyme solution (total volume 35 mL).

7. Although enzymatic defolliculation is primarily targeting collagen fibers comprising the theca, the most effective products are not pure collagenase but a preparation that contains a mixture of proteases. Collagenase type 1 is a crude product containing other proteases in addition to collagenases. Liberase is preferred for many applications because it contains highly purified collagenases, has significantly higher specific activities, and is more consistent between lots.
8. Check digestion every 30 min. Enzymatic overtreatment can irreversibly damage the oocytes, which begin to undergo apoptosis, with proteolytic mechanisms turning oocytes white and promoting swelling. Defolliculation takes approximately 1–3 h. Digesting at a higher temperature (25–30 °C) accelerates the process; however that increment can activate heat-shock processes [26].
9. Stage V-VI oocytes are released first by enzymatic treatment. If earlier stage oocytes are desired, the clumps of tissue can be further digested. Digestion is terminated by pipetting the freed oocytes into 150 mm culture dishes containing 100 mL MBS, followed by two 100 mL MBS washes.
10. Defolliculated oocytes are cultured overnight in MBS at 18 °C to allow recovery from the effects of the enzymatic digestion. The oocytes can then be cultured in O-R2 medium for up to 3 or 4 days at 18 °C, changing the medium twice daily. The addition of antibiotics is useful to maintain healthy oocytes during longer term culturing.
11. Healthy stage I/early stage II oocytes will have a white, compact Bb; late stage II/early stage III oocytes will have a disk/cone of Bb material attached to the vegetal cortex.
12. Obtain ovarian tissue ideally from young female Xenopus frogs that measure approximately 5 cm from the top of the head to the cloaca.
13. Set up centrifuge for 4 °C (will likely have to turn vacuum on to cool down centrifuge).
14. If there are greater than 700–800 μL of oocytes, split them into two (or more) sterile 1.5 mL microfuge tubes.
15. To avoid bubbles, place the beveled end of the needle against the wall at the bottom of the tube; draw the plunger until there is only enough volume left in the tube to cover the needle bleb (i.e., do not draw up any air).
16. The mitochondria upper band together with a ball of material floating on top of it is collected as the mitochondrial cloud or Balbiani body material. Usually, the upper band and the ball are coherent.

17. Moving the petri dish in a circular motion will help move the oocytes to the center for easy collection/transfer.
18. The Bb may stick to the forceps and pulled glass needle; be patient and gently maneuver the Bb between the forceps and needle until it is freely floating in the OR2. If the Bb is not freed within 5–10 min, discard that oocyte and move on to the next.
19. It works well to isolate ~3–10 Bbs and then transfer them to the collection tube.
20. Recentrifugation is to ensure that the Bbs that have been collected are at the bottom of the tube and thus will not be accidently removed upon adding more Bbs.
21. Keep track of the number of isolated Bbs. As a reference, after 8–10 h it is possible to collect approximately 100–200 Bbs.
22. If ~100 Bbs or more are collected, a pellet should be visible; if not, spin for an additional 15 min.
23. In Boke et al., 2016 [27], the authors manually isolate Xenopus Bbs using a cytoskeleton-stabilizing buffer (CSB) (10 mM Pipes buffer, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM glucose monohydrate). An explanatory video can be found in http://www.cell.com/cell/fulltext/S0092-8674(16)30859-5.

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Chapter 16

Visualizing the Balbiani Body in Zebrafish Oocytes

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Abstract

Approaches to visualize the Balbiani body of zebrafish primary oocytes using protein, RNA, and mitochondrial markers are described. The method involves isolation, histology, staining, and microscopic examination of early zebrafish oocytes. These techniques can be applied to visualize gene products that are localized to the Balbiani body, and when applied to mutants can be used to decipher molecular and genetic pathways acting in Balbiani body development in early oocytes.

Key words Oocyte, Balbiani body, Mitochondria, RNA and protein localization, Primary oocytes, Buckyball, Immunohistochemistry, Microscopy, Histology

1 Introduction

In vertebrates the first axis to form is the animal-vegetal axis. Although the animal-vegetal axis forms during oogenesis, the genes that are necessary to establish and maintain the animal-vegetal axis are only beginning to be illuminated because the maternal regulators of animal-vegetal axis formation act during early oogenesis, and thus are only accessible through genetic approaches that disrupt maternal gene products and allow examination of asymmetries established in early oogenesis (reviewed in [1]). The Balbiani body has been detected in the primary oocytes of all oocytes examined thus far (reviewed in [2–4]). It is a non-membrane-bound compartment, comprised of organelles, including mitochondria, endoplasmic reticulum, and a select subset of an oocyte's RNAs and proteins [4]. Although the relationship between the Balbiani body and the mammalian oocyte axis has not been reported, this structure is an early indicator of the animal-vegetal axis in nonmammalian vertebrates [4]. The earliest stages of Balbiani body formation are apparent by the localization of essential proteins such as Buckyball, a protein that is required for oocyte polarity and assembly of the Balbiani body [5–9]. During the first meiotic prophase, the components of the Balbiani body are

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_16,

found near the nucleus [5, 9] and reviewed in [4]. Buckyball, once translated, promotes a process that gathers Balbiani body components into a discrete subcellular space or compartment [5, 7–9]. In mutants that lack functional Buckyball protein, no Balbiani body forms [7, 8]. As the oocyte progresses through the first meiotic prophase, the Balbiani body is relocated from a position adjacent to the nuclear membrane to the cell cortex by a process that requires the protein Microtubule-actin cross-linking factor-1 (MacF1) [10, 11]. Prior to diakinesis of the first meiosis the Balbiani body dissociates. In mutants lacking normal MacF1 function, the Balbiani body becomes too large and persists into later stages of oogenesis [10, 11]. The consequence of Balbiani body defects can be seen in the lack of an animal-vegetal axis in the embryos produced by mutant mothers [7, 12–14], but visualization of the Balbiani body to determine the nature of the Balbiani defect requires immunohistochemistry and labeling of Balbiani body structures. Here we present methods to isolate ovaries and oocytes and to label and visualize components of the Balbiani body of zebrafish oocytes.

2 Materials

Solutions should be made before the start of the experiments. Solutions can be prepared and stored at room temperature, unless noted otherwise. *See* Table 1 for a summary of specific reagents and equipment.

2.1 Prepare Adult or Juvenile Fish for Dissection

1. Crossing tanks.
2. Embryo media: 60 μg/mL Instant Ocean sea salts, pH 7.0: To prepare embryo media, dissolve 0.6 g of instant ocean sea salts in 1 L of dH_2O. Once the salts have been dissolved, adjust the pH by slowly adding sodium bicarbonate and measuring the pH until it reaches pH 7.0 [15].
3. Tricaine stock solution: 0.4% (w/v) Tricaine, 21 mM Tris base, pH 7.4: Tricaine methanesulfonate is an irritant and should be measured and prepared in the hood or with appropriate ventilation and protective equipment (e.g., gloves, lab coat). Working in a fume hood, measure and dissolve 0.4 g of tricaine in 100 mL of 21 mM Tris buffer (97.9 mL of dH_2O and 2.1 mL of 1 M Tris pH 9.0). Once the tricaine is fully dissolved, adjust the pH to 7.4 by slowly adding 2 M NaOH and measuring the pH until it reaches pH 7.4. When using tricaine for anesthesia, dilute 9 mL of tricaine stock in 200 mL of embryo media (*see* **Note 1**).
4. L-15 media (commercially available): Store at 4 °C.
5. Poly-L-lysine: 0.01% (w/v), pH 7.0.

Table 1
List of specific reagents and equipment

Name of the reagent
Instant Ocean sea salt
Tricaine (ethyl 3-aminobenzoate methanesulfonate, MS-222)
L-15 media (mod.)
Collagenase[a]
Penicillin-streptomycin solution
Gentamicin
Hepes
FBS
MitoTracker Green FM
Rhodamin-dextrane
Vectashield Antifade Mounting Medium with DAPI
$DiOC_6$
Proteinase-K solution
Deionized formamide
Heparin sodium salt from porcine intestinal mucosa
Ribonucleic acid, transfer from wheat germ
50% Dextran sulfate solution
Goat serum
Bovine serum albumin
Anti-digoxigenin-AP Fab fragments
BM-Purple
Dissecting mat
Fine-tip Pasteur pipette
Glass-bottom dish
12-Well Transwell plate with insert
Dissecting scope
Axio Observer (Zeiss)

[a]Enzyme activity needs to be tested empirically

6. Poly-L-lysine-coated 6-well plates: Pipette poly-L-lysine into wells of 6-well plate. Take care to cover the bottom completely as well as the sides of the wells. Let the poly-L-lysine sit for

5 min. Remove the excess poly-L-lysine and leave to dry. Store the plates at room temperature and keep protected from dust.

7. OR2 buffer: 0.1 mM Na_2PO_4, 5 mM Hepes, 1 mM $CaCl_2$, 2.5 mM KCl, 8.25 mM NaCl, 1 mM $MgCl_2$, pH 7.4.

   This is the buffer that will be used if isolating oocytes. To prepare 1 L, combine 1 mL 100 mM Na_2PO_4, 5 mL 1 M Hepes, 1 mL 1 M $CaCl_2$, 1 mL 2.5 M KCl, 100 mL 82.5 mM NaCl, and 1 mL 1 M $MgCl_2$, and add dH_2O. This buffer can be warmed in the incubator prior to use.
8. Collagenase, Type I: 1 mg/mL collagenase, pH 7.5

   Store at 4 °C

   The enzyme activity can vary between batches and should be tested empirically. Store at 4 °C
9. M199 Media (optional): Store at 4 °C.
10. Kimwipes.
11. 70% Ethanol.
12. Dissecting mat.
13. Petri dish.
14. Dissecting microscope.

2.2 Dissections

1. Razor blades.
2. Dumont #5 forceps.
3. Vannas Spring Scissors.

2.3 Oocyte Dissociation

1. L-15 Media: Store at 4 °C.
2. OR2 Buffer with Collagenase: 15 mg/mL Collagenase, 0.1 mM Na_2PO_4, 5 mM Hepes, 1 mM $CaCl_2$, 2.5 mM KCl, 8.25 mM NaCl, 1 mM $MgCl_2$, pH 7.4.

   Prepare this on the day of use. Store at 4 °C for up to a week.
3. Shaker.
4. Disposable glass Pasteur pipets.
5. Cell culture CO_2 incubator.

2.4 Oocyte Sorting and Culture

1. L-15 Media: Store at 4 °C.
2. Dumont #5 forceps.
3. Falcon™ Polystyrene Microplates, 6 wells or 24 wells.
4. Fetal bovine serum (FBS): Store at 4 °C.
5. Penicillin-streptomycin: 5000 U/mL, pH 7.0.

   Store at −20 °C.
6. Gentamycin: 10 mg/mL.

7. $L15^{+}$ media: 50 U Penicillin-streptomycin, 2 mg/mL gentamycin, 15 mM Hepes pH 7.5, 20% FBS or ovary extract, in L-15 media.

   Store at 4 °C.
8. Ovary extract (optional): Recommended if you will be culturing oocytes. Dissect ovary and flash freeze in a 1.5 mL tube by submerging the tube liquid nitrogen until frozen (the tissue will freeze quickly and turn whitish in color) (*see* **Note 2**). Add 500 μL L-15 or m199 media to frozen ovary tissue, thaw on ice, and homogenize using a motorized pestle or sonicate the tissue. Once homogenized, add an additional 500 μL L-15 or m199 media, and then centrifuge for 30 min at maximum speed and 4 °C. Carefully remove the supernatant which contains the ovary extract and discard the pelleted material. Store at 4 °C.
9. Cell culture CO_2 incubator (only needed if culturing oocytes).

2.5 Visualizing Mitochondria

1. MitoTracker™ Red CMXRos (optional): Store at −20 °C and keep protected from light by wrapping in foil.
2. 10× PBS: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4.

   Dissolve 80 g of NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 mL of dH_2O. Adjust the pH to 7.4 by slowly adding 2 M HCl and measuring in between each addition. Bring the volume to 1 L with dH_2O. Autoclave to sterilize. This can be diluted to make 1× PBS.
3. 4% Paraformaldehyde (PFA): 4% (w/v) PFA, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

   PFA is hazardous and should be measured and prepared in the hood with appropriate ventilation and protective equipment (e.g., gloves, lab coat). Heat 80 mL of 1× PBS to 50 °C; take care that it does not boil. Dissolve 4 g of PFA in the PBS (*see* **Note 3**). Cool to room temperature. Adjust pH to 7.4 with 2 M HCl. Bring to volume with 1× PBS. Store at 4 °C (*see* **Note 4**). Discard according to institutional guidelines.
4. 100% Methanol (optional): Discard according to institutional guidelines.
5. Compound microscope.

2.6 $DiOC_6$ Labeling to Visualize Mitochondria and ER

1. 1.5 mL Microcentrifuge tubes.
2. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

   Dilute 10× PBS (see above for details) with dH_2O.
3. $DiOC_6$: 100 mg/mL.

Wear appropriate protective gear (gloves, eye protection) as DMSO readily absorbs through the skin, and solubilize DiOC6 by adding 1 mL dimethyl sulfoxide (DMSO) to $DiOC_6$. Vortex to mix. Store at 4 °C and keep the solution protected from light.

4. Vectashield Antifade Mounting Medium with DAPI: Store at 4 °C and protect from the light.

2.7 Immunohistochemistry

1. 1.5 mL Microcentrifuge tubes.
2. Shaker.
3. 4% PFA (see above for details): Store at 4 °C. Discard according to institutional guidelines.
4. 1× PBS (described above).
5. 100% Methanol (optional): Discard according to institutional guidelines.
6. Proteinase-K: 20 mg/mL, pH 7.0–7.5.

   Store at −20 °C.
7. PBS + Tween: 0.1% (w/v) Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

   Mix 50 μL of Tween-20 in 50 mL of 1× PBS.
8. Goat serum: Store at 4 °C (*see* **Note 5**).
9. Blocking solution: 5% (w/v) goat serum, 0.1% (w/v) Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

   PBS + Tween with 5% goat serum. Add 50 μL of goat serum per 950 μL of PBS + Tween. This solution should be made fresh for each experiment and stored at 4 °C (*see* **Note 6**).
10. Primary antibody: Aliquot and store according to the manufacturer's instructions.
11. Freezer box for 1.5 mL tubes.
12. Tin foil (optional).
13. Secondary Antibody: Aliquot and store according to the manufacturer's instructions. Protect from light.
14. Vectashield Antifade Mounting Medium with DAPI: Store at 4 °C, and protect from light.
15. Frosted microscope slides.
16. 18 mm × 18 mm Cover glass.
17. 25 mm × 25 mm Cover glass.
18. Super glue for precision application.
19. Vaseline.

2.8 In Situ Hybridization

1. 1.5 mL Microcentrifuge tubes.
2. 4% PFA: Store at 4 °C. Discard according to institutional guidelines.
3. 100% Methanol: Discard according to institutional guidelines.
4. 1× PBS.
5. Proteinase-K: Proteinase-K: 20 mg/mL, pH 7.0–7.4.

   Store at −20 °C.
6. PBS + Tween.
7. Water bath set to 65 °C for high stringency or 55–60 °C for lower stringency.
8. 20× SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0.

   Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 1 L of dH_2O. Adjust pH to 7.0 by slowly adding 2 M HCl. Measure between each addition of 2 M HCl. This stock can be diluted to make 2× and 0.2× concentrations.
9. Deionized formamide: 99% Formamide. Store at 4 °C (*see* **Note 7**).
10. Heparin sodium salt from porcine intestinal mucosa (heparin): 50 mg/mL Heparin.
11. Ribonucleic acid, transfer from wheat germ (tRNA): 50 mg/mL tRNA.

    Resuspend tRNA in dH_2O and then "clean up" with phenol/chloroform extractions (*see* **Note 8**). In a 1.5 mL tube add 50 µL of the resuspended tRNA and 500 µL of phenol/chloroform. Centrifuge for 30 min at maximum speed and 4 °C. Remove the clear aqueous layer. Repeat the phenol/chloroform extractions three times. Precipitate with ethanol, resuspend the tRNA, measure the concentration, and adjust as needed to a final concentration of 50 mg/mL.
12. Hyb − Buffer: 50% Deionized formamide, 5× SSC, 0.1% Tween-20, adjust to pH 6.0.

    Store at −20 °C.
13. Hyb + Buffer: 50% Deionized formamide, 5× SSC, 0.1% Tween-20, 50 µg/mL heparin, 500 µg/mL tRNA, pH 6.0.

    Store at −20 °C.
14. Antisense RNA probe solution: Probes should be prepared as in [16]. Dilute antisense RNA probe in Hyb + Buffer as recommended. Store at −20 °C.
15. Bovine serum albumin (BSA).
16. Blocking buffer: PBS + Tween, 2% goat serum, 2 mg/mL BSA.

    Combine 49 mL PBS + Tween with 1 mL goat serum and 100 mg BSA. This solution should be made fresh for each experiment and stored at 4 °C (*see* **Note 9**).

17. Anti-digoxigenin-AP Fab fragments (for Dig-labeled RNA probes): Store at 4 °C.
18. Alkaline Tris buffer: 100 mM Tris–HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20, pH 7.4.
19. BM-Purple: Store at 4 °C.
20. Frosted microscope slides.
21. 18 mm × 18 mm Cover glass.
22. 25 mm × 25 mm Cover glass.
23. Super glue for precision application.
24. Vaseline.

3 Methods

3.1 Prepare Female Fish

1. For adult females, 2 days prior to dissection, set up zebrafish according to standard crossing procedures to clear maturation-competent oocytes (*see* **Note 10**).
2. On the day of dissection, prepare tricaine anesthesia (AVMA Guidelines on Euthanasia, June 2007) as indicated above.
3. Prewarm a 50 mL aliquot of L-15 media at 32 °C if you plan to culture the ovary tissue or oocytes after dissection. Prepare a poly-L-lysine-coated 6-well plate for tissue dissociation. Fill the first well with 3–4 mL L-15 media (*see* **Note 11**). Prepare the next well by adding 3 mL OR2 buffer. To dissociate follicle cells (somatic or non-germline cells) from oocytes, add 25 μL Collagenase (15 mg/mL in OR2 buffer). Fill the third well with 3–4 mL L-15 media or M199 media.
4. In the morning, anesthetize female in tricaine solution [15] (*see* **Note 12**). If culturing the oocytes, first disinfect the fish skin by gently wiping the sides of the body with a kimwipe that has been moistened with 70% ethanol. Transfer the fish to a dissecting mat or a petri dish lid and position the fish on its side under a dissecting microscope (Fig. 1).

3.2 Ovary Dissection

1. Decapitate female with razor blade, by cutting just posterior to the operculum (Fig. 1, marked with an arrow). Turn the fish to position the ventral side (belly) upward, and using forceps or dissecting scissors carefully open the skin from head to anal fin (Fig. 1).
2. If culturing oocytes take care to avoid contaminating your sample by puncturing the intestine, which is positioned just beneath the ventral muscles as you grasp the intestine near the anus and pull it slowly upward and toward the rostral end to remove all organs except the two ovaries and swim bladder, which are visible within the body cavity (Fig. 1).

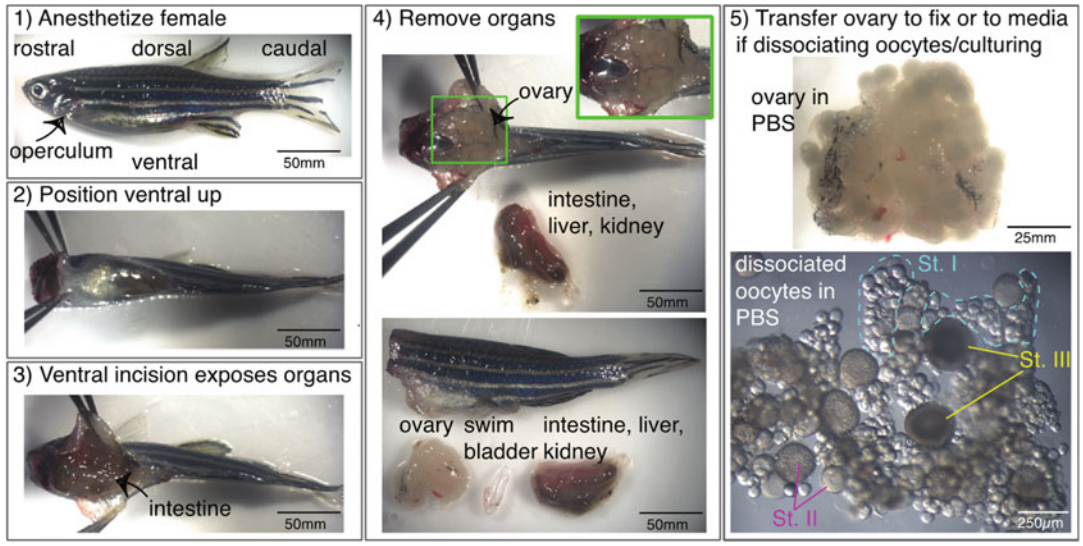

Fig. 1 Ovary dissections. (1) Anesthetized adult female fish positioned laterally with dorsal up on a petri dish, under a dissection scope. (2) Decapitated fish positioned using forceps to orient with ventral side up. (3) Using the forceps, gently tear the skin to open the belly. The intestines and other organs are now exposed. (4) Organs are gently removed to expose the ovaries, which extend to the dorsal aspect of the body cavity. Inset shows the swim bladder (rostral end of the opening) and ovaries within the body cavity. Using the forceps and swim bladder, the ovaries are separated from the body walls and removed. Displayed are the ovaries, swim bladder, and other organs for reference. (5) Various size and stage oocytes can be seen in the whole ovary tissue (top). Black is pigmented tissue that is found proximal to the ovary. Stage I oocytes are shown, outlined in blue (these are oocytes which have a Balbiani body). Stage II and stage III oocytes are shown with magenta and yellow lines, respectively. Stage I oocytes appear more colorless, while stage II and stage III appear more yellow in color

3. Use forceps to gently separate the ovaries from the body wall. Return the fish to a lateral position, and using the swim bladder as a cushion carefully remove the ovaries (Fig. 1) (*see* **Note 13**).

3.3 Oocyte Dissociation

1. Transfer the ovary into L-15 media in the prepared 6-well plate (*see* **Note 14**).
2. To dissociate oocytes from the follicle cells, transfer the ovary from L-15 media to OR2 buffer with Collagenase and incubate for 30 min at RT. Apply moderate agitation during the incubation period using a pipet or on a shaker.
3. Transfer the ovary to a new well that has been previously filled with L-15 media. At this point you will begin to observe loose late-stage oocytes and follicle cells that have separated from the bulk tissue. To wash the dissociated cells incubate the dissected ovary tissue for 10 min on a shaker. Remove the L-15 media with a fine-tip Pasteur pipette and add 3–4 mL L-15 media that has been pre-warmed to 32 °C. Repeat this step two additional times to remove any residual collagenase and the dissociated

follicle cells. Allow the dissociated oocytes to recover in a cell culture incubator set to 32–37 °C and 8–14% CO_2 for at least 1 h.

3.4 Sorting Oocyte Stages and Culture

1. Change the L-15 media again and then use forceps to manually dissociate the oocytes. Any mature, large yolk-containing oocytes should be eliminated at this stage as they will begin to change color from translucent to grey as they deteriorate.
2. Oocytes can be sorted into groups based on size and stage-specific features (Fig. 1). Place sorted oocytes into wells that contain fresh L-15 media (depending on the number of cells to be collected 6-well plates or 24-well plates are convenient for this step). Stage III and IV oocytes can be manually plucked and sorted first. Smaller oocytes (stage I and II) tend to remain encased in follicle cells and are closely associated with blood vessels. These somatic cells can be stripped away from the oocytes using forceps. Stage II oocytes, which no longer have Balbiani bodies, can be distinguished from primary oocytes by their yellow hue and the presence of cortical granules (Fig. 1) (*see* **Note 15**).
3. Oocytes can be cultured for several days to weeks in a cell culture incubator in $L15^{+}$ media (*see* **Note 16**).

3.5 Visualizing Balbiani Body Components

Depending on the assay oocytes can be directly monitored in vivo to visualize the expression of a transgenic reporter or using vital dies to label organelles, such as mitochondria and endoplasmic reticulum (e.g., $DiOC_6$ or MitoTracker) (Fig. 2). Alternatively, oocytes can be fixed in 4% PFA or methanol for in situ hybridization or immunohistochemistry to detect Balbiani body-localized RNAs and proteins, respectively.

3.5.1 Visualizing Mitochondria

1. Dilute $DiOC_6$ 1:5000 in 1× PBS and incubate live ovary in 1 mL at room temperature for 30 min (*see* **Note 17**).
2. Wash the tissue with PBS for 30 min; repeat this step six times.
3. Tissue can now be mounted with Vectashield Antifade Mounting Medium with DAPI (if visualization of nuclei and staging of oocytes are required), or in Vectashield Antifade Mounting Medium (if nuclear labeling is not necessary). The tissue will be ready to image within 30 min; however, the signal-to-noise ratios are significantly improved with longer incubations. Fixed tissues can be stored in 1× PBS for several weeks at 4 °C and protected from the light.
4. While tissue is in Vectashield Antifade Mounting Medium with DAPI, prepare slides for imaging. Three-bridged slides are recommended for ovary tissue. Using super glue, glue 18 mm × 18 mm coverslips to the glass slide. Repeat so that

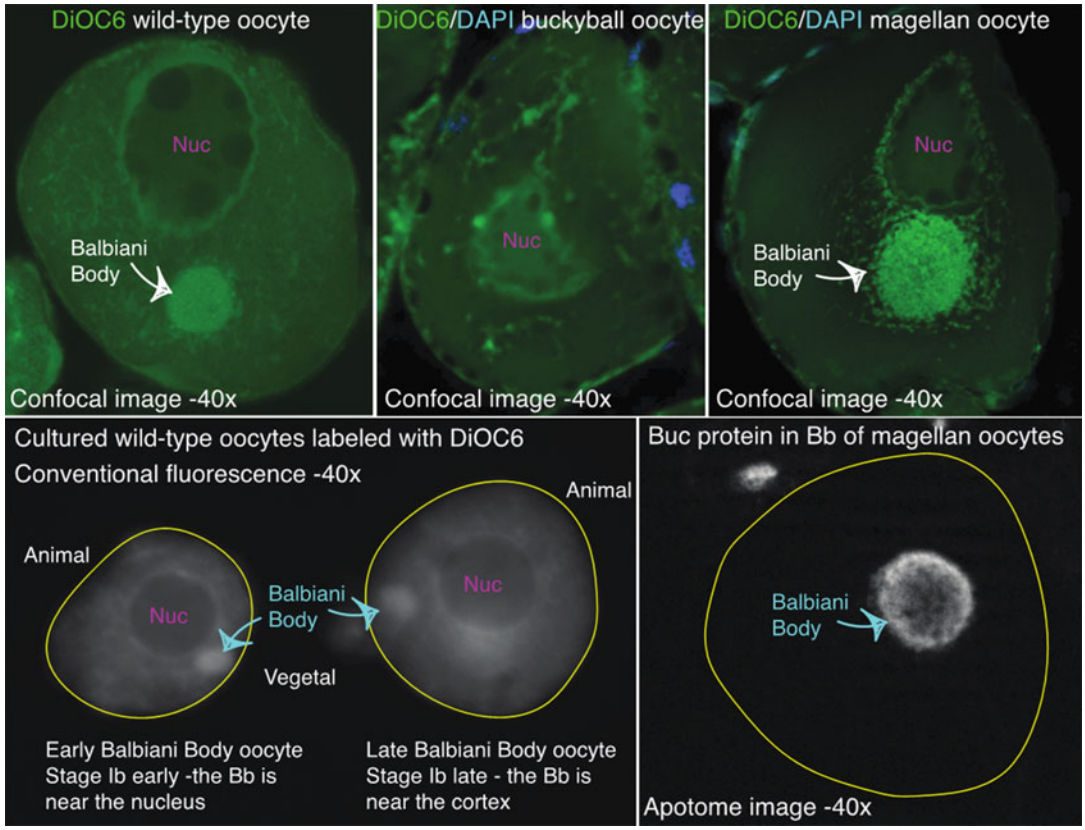

Fig. 2 Examples of $DiOC_6$ staining and Buckyball antibody labeling using different imaging platforms. Top panels show confocal images of $DiOC_6$ -labeled Balbiani bodies of WT (top left), lack of Balbiani body in *buckyball* mutant oocytes (top middle), and the enlarged Balbiani body that fails to relocate in *magellan* mutant oocytes (top right). Conventional fluorescence image of $DiOC_6$-stained WT oocytes (bottom left). The Balbiani body is found next to the nucleus in early-stage Ib oocytes and relocates to a position near the cortex of the oocyte in late-stage Ib oocytes. Apotome image of Buckyball protein in the enlarged Balbiani body of a *magellan* mutant oocyte (bottom right). The greatest subcellular resolution is achieved with confocal microscopy, followed by Apotome. Conventional fluorescence can be used to detect the presence and size of the Balbiani body but provides less subcellular detail

3–18 mm × 18 mm coverslips are glued on top of one another. There should be two stacks of coverslips to make a gap in between; it is recommended that the gap does not exceed 18 mm in distance.

5. Place Vaseline on the top and bottom of the gap created by the coverslip stacks to seal the area.
6. Place ovary in the gap, and include a drop or enough Vectashield to cover the tissue and keep the sample moist (*see* **Note 18**).

7. Position a 25 mm × 25 mm coverslip over the coverslip stacks and gap to close it off. Tissue is now mounted and can be imaged.
8. Fluorescence and bright-field images were acquired sequentially using an Axio Observer compound microscope equipped with Apotome and Axiovision Rel4.6 software (Zeiss). Composite figures were prepared using ImageJ (version 1.48k) and Adobe Photoshop and Illustrator software (version CS4).

3.5.2 Immunohistochemistry

1. For whole-mount analysis, ovary tissue should be fixed in at least 1 mL of 4% PFA (or up to 10× the tissue volume) and incubated overnight at 4 °C on a shaker. 1.5–2 mL tubes are suitable to use for the entire experiment (*see* **Note 19**).
2. The next day, remove the 4% PFA and wash samples in 1× PBS for 5 min. Repeat PBS washes five times. At this point the samples can be transferred to 100% methanol and stored at −20 °C for future use.
3. After PBS washes, the tissue is permeabilized with proteinase-K that has been diluted 1:2000 in 1× PBS. Add proteinase-K PBS to the tissue and incubate at room temperature for 15 min (*see* **Note 20**).
4. Remove proteinase-K PBS. Wash with PBS + Tween for 5 min, and repeat PBS + Tween washes three times.
5. Block samples in blocking solution for 2–4 h, at room temperature on a shaker.
6. Incubate with primary antibody overnight, at 4 °C on a shaker. Primary antibody should be diluted according to the manufacturer's recommendation in blocking solution (*see* **Note 21**).
7. Remove the primary antibody. The solution can be saved and reused up to three times or discarded if no longer needed.
8. Wash the tissue in PBS + Tween for 30 min, at room temperature on a shaker. Repeat this step six times.
9. Secondary antibody should be diluted according to the manufacturer's recommendation. Incubate with fluorescent-conjugated secondary antibody overnight, at 4 °C on a shaker. For the rest of the experiment, the tissue should be protected from the light to prevent bleaching of the fluorescent signal. This can be done by keeping samples in a dark box or wrapping in foil when they are not being handled (*see* **Note 22**).
10. Remove secondary antibody and discard solution.
11. Wash the tissue with PBS + Tween for 30 min; repeat this step six times.
12. Add enough Vectashield Antifade Mounting Medium with DAPI to cover the tissue. The tissue will be ready to image

within 30 min; however, signal-to-noise ratios are significantly improved with longer incubations. Samples can be stored at 4 °C in Vectashield Antifade Mounting Medium with DAPI for several weeks.

13. The tissue should be mounted on three-bridged slides (described above) for imaging. Tissue can also be stored in PBS at 4 °C and protected from the light for several weeks until ready to image.

3.5.3 In Situ Hybridization

1. For whole-mount analysis, dissected ovary tissue should be fixed in 1 mL of 4% PFA (or up to 10× the tissue volume) and incubated overnight at 4 °C on a shaker. 1.5–2 mL tubes are suitable to use for the entire experiment (*see* **Note 23**).
2. The next day, remove the 4% PFA and wash samples in 1× PBS for 5 min. Repeat PBS washes five times. At this point the samples can be transferred to 100% methanol and stored at −20 °C indefinitely for future use.
3. After PBS washes, the tissue is permeabilized with proteinase-K that has been diluted 1:2000 in 1× PBS. Add proteinase-K PBS to the tissue and incubate at room temperature for 15 min (*see* **Note 24**).
4. Remove proteinase-K PBS. Inactivate the proteinase-K, by fixing the tissue in 4% PFA at room temperature for 20 min.
5. Wash samples in PBS + Tween for 5 min. Repeat PBS + Tween washes five times.
6. Incubate the samples in Hyb + buffer at 65 °C in a water bath for 2–4 h.
7. Remove Hyb + buffer and add 500 μL antisense RNA probe solution. Incubate overnight at 65 °C in a water bath.
8. Remove probe solution. Probe solution can be saved and reused, or discarded if no longer needed.
9. Rinse samples in Hyb − buffer.
10. Wash samples in a solution made of 75% Hyb − buffer and 25% 2× SSC at 65 °C for 15 min.
11. Wash samples in a solution made of 50% Hyb − buffer and 50% 2× SSC at 65 °C for 15 min.
12. Wash samples in a solution made of 25% Hyb − buffer and 75% 2× SSC at 65 °C for 15 min.
13. Wash samples with 100% 2× SSC at 65 °C for 15 min.
14. Wash samples with 100% 2× SSC at 65 °C for 30 min. Repeat this wash twice.
15. Wash samples in a solution made of 75% 0.2× SSC and 25% PBS + Tween at room temperature for 10 min.

16. Wash samples in a solution made of 50% 0.2× SSC and 50% PBS + Tween at room temperature for 10 min.
17. Wash samples in a solution made of 25% 0.2× SSC and 75% PBS + Tween at room temperature for 10 min.
18. Wash samples with 100% PBS + Tween at room temperature for 10 min.
19. Incubate samples in blocking solution at room temperature for 2–4 h.
20. Replace blocking solution and dilute anti-digoxigenin-AP Fab fragments 1:1000 in fresh blocking solution. Incubate over night at 4 °C on a shaker.
21. Remove blocking solution with anti-digoxigenin-AP Fab fragments (to detect digoxigenin-labeled probes), and rinse samples with PBS + Tween.
22. Wash samples in PBS + Tween for 15 min. Repeat PBS + Tween washes six times.
23. Wash samples in alkaline Tris buffer for 5 min. Repeat alkaline Tris buffer washes three times.
24. Add 500 μL of BM-Purple to samples. Tissue should be protected from the light to protect the light-sensitive substrate during development. This can be done by keeping samples in a dark box or wrapping in foil when they are not being handled. Samples should be monitored (*see* **Note 24**).
25. To stop the colorimetric reaction, remove the BM-Purple solution and wash samples with PBS pH5.5 + 1mM EDTA for 5 min. Repeat washes five times.
26. The tissue should be mounted on three-bridged slides (described above) for imaging. Tissue can also be stored in PBS at 4 °C and protected from the light for several weeks until ready to image.

4 Notes

1. Tricaine stock solution can be made at any time and frozen as 9 mL aliquots in 15 mL conical tubes. Aliquots can then be taken out and thawed as needed.
2. For freezing tissue in liquid nitrogen, use screw-top version of 1.5 mL tubes (Thermo Scientific, 3467) to ensure that the tube remains shut during freezing. Non-locking tops may open during freezing due to the extreme temperature change. When this occurs liquid nitrogen can come in contact with the tissue and damage it.

3. 2 M NaOH may be needed to help the PFA dissolve. Adding a few drops at a time helps to bring the PFA into solution.
4. 4% PFA can be made at any time and frozen as aliquots. Aliquots can then be thawed as needed.
5. Goat serum will expire after a few weeks at 4 °C. Expiration will become noticeable by the presence of precipitates in the solution. Goat serum can be frozen as aliquots, and thawed as needed to preserve it.
6. Blocking solution with 5% goat serum can be stored at 4 °C for a week. Make sure that solution has not expired before using. Blocking solution with precipitates should not be used.
7. We recommend storing deionized formamide in aliquots and thawing as needed.
8. Use caution when working with phenol/chloroform. Personal protection equipment such as eye goggles, gloves, and lab coat should be worn while handling phenol/chloroform. Phenol/chloroform should be handled under a fume hood with appropriate ventilation.
9. Blocking solution with 2% goat serum and 2 mg/mL BSA can be stored at 4 °C for a few weeks. Goat serum may expire and the presences of precipitation in solution may become apparent. Make sure that the solution has not expired before using.
10. Mating clears mature and maturation-competent oocytes form the ovary. Although this step is not necessary to proceed with dissecting the ovaries, clearing the maturation-competent oocytes effectively enriches for early-stage oocytes in the ovary making it easier to find Balbiani body-stage oocytes.
11. As an alternative to L-15 media, M199 media can be used for short-term culture of oocytes.
12. You will know when females are anesthetized, as they will no longer swim. They will rest at the bottom and exhibit only slow movement of the operculum.
13. If oocytes do not need to be cultured you may immediately proceed to fixing the ovary in 4% PFA or methanol.
14. Ideally cells should be isolated as soon as possible following dissection, but if necessary the dissected tissues can remain in L-15 media without adverse effects for up to 1 h if you need to collect oocytes from several ovaries consecutively, or if you also need to collect other organs or tissue for other analysis, such as genotyping of your fish.
15. Balbiani bodies are present in stage I oocytes, but are absent by stage II oocytes. Therefore, for analysis of Balbiani body formation primary oocytes should be sorted from adults or younger females (35dpf), which lack mature oocytes, to enrich for primary oocytes. To determine if the Balbiani body persists

longer than is normal for wild type, as occurs in *magellan* mutants (Fig. 2) [10, 11] stage II oocytes can be selected for analysis based on the presence of cortical granules (*see* Fig. 1).

16. For sorted oocytes in culture, the media should be changed every second day or more frequently if indicated by the pH indicator in the media (the pink media will become orange/yellow in color). Add 3 mL $L15^{+}$ media to each well if using 6-well plates and add 400 μL to each well if using 24-well plates.
17. $DiOC_6$ labeling can be performed on either live of fixed tissue. If you are using MitoTracker, labeling must be done in live tissue. For best results, ovary tissue should be labeled immediately after dissection and visualized within 2 h post-dissection. Some mitochondrial dyes are fix stable. For these dyes labeling is performed on living tissues immediately after dissection and then the labeled tissues are fixed. It is also possible to culture oocytes for several days to weeks; however, samples should always be analyzed at comparable stages with respect to hours post-dissection. Be sure to check manufacture specifications to determine if the dyes should be applied to living or fixed tissue and if they are fix stable post-labeling. For directions on how to fix ovary tissue *see* **steps 1** and **2** of Subheadings 3.5.2 or 3.5.3.
18. Tissue can be removed from the 1.5 mL tubes with forceps. If the forceps cannot reach the tissue, gently tap the tube upside so that the tissue moves closer to the opening of the tube.
19. Immunohistochemistry is performed over the course of multiple days, so plan accordingly.
20. For the permeabilization step, watch the time carefully. Do not exceed a 15-min incubation as the tissue can be degraded too much, target proteins can be destroyed, and the labeling compromised.
21. If using multiple primary antibodies, ensure that the host species for each antibody is unique to prevent cross-reactivity.

    If using multiple secondary antibodies, ensure that the excitation wavelengths of the secondary antibodies are not similar to prevent bleed-through of signal to another channel. Take note of which secondary antibodies correspond to which primary for reference when imaging.
22. In situ hybridization is an experiment that is done over the course of multiple days, so plan accordingly.
23. For the permeabilization step, watch the time carefully. Do not exceed a 15-min incubation as the tissue can become compromised and target RNAs can be destroyed.
24. Precipitates of the colorimetric reaction can appear as quickly as 30 min; however, developing times can range from 1 to 5 h. Monitor your samples for color development every 15 min.

During color development samples must be monitored to achieve robust signals and avoid overdevelopment. You want to ensure that all expression is visible but also limit the amount of background that is present. Variation in development times can be ascribed to differences in probes and the abundance of the target RNA expression.

Acknowledgments

This work was partially supported by the National Institutes of Health R01GM089979.

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Chapter 17

Isolation of Zebrafish Balbiani Bodies for Proteomic Analysis

Allison Jamieson-Lucy and Mary C. Mullins

Abstract

Proteomic characterization of isolated organelles can provide insight into the functional components of the structure and novel targets for further testing. Germplasm in developing oocytes is difficult to isolate for protein identification because not all types of germplasm are stable outside of the cytoplasm. In zebrafish, the Balbiani body forms a proteinaceous aggregate that contains the germplasm and we found is stable outside of the oocyte. Here we present a manual isolation protocol that collects intact Balbiani bodies from stage I zebrafish oocytes. We lysed oocytes by passing them through a syringe, and then used a fine injection needle to wick up Balbiani bodies by capillary action with minimal buffer solution. Using this protocol we collected sufficient material for proteomic analysis of the zebrafish Balbiani body.

Key words Balbiani body, Germplasm, Proteomics, Isolation, Oocytes

1 Introduction

In animals ranging from roundworms to amphibians, germ cells are specified by a substance within the oocyte called germplasm [1, 2]. The germplasm is electron-dense material containing RNA and protein, which is separated from the rest of the oocyte cytoplasm by a phase transition: either liquid-liquid or liquid-solid [3]. In zebrafish, the germplasm is carried by the Balbiani body, which is a solid proteinaceous aggregate containing ribonucleoprotein particles, mitochondria, endoplasmic reticulum, and other membrane-bound organelles [4]. The Balbiani body is essential for producing fertile offspring [5–8], but only a few genes are known to act in Balbiani body formation or its maintenance. Proteomics can expand our understanding of germ granules by providing novel protein targets for functional studies, as well as global insight into the proteins enriched in these structures.

Several methods have been used to purify different types of non-membrane-bound organelles. Neurofibrillary tangles and deposits of amyloid-ß, hallmark lesions in Alzheimer's disease,

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_17,

have been collected from brain tissue for proteomic analysis using a variety of methods including gradient density centrifugation, laser capture microdissection, and sequential centrifugation [9–12]. Stress granule cores have also been isolated from cultured cells using sequential centrifugation followed by purification by immunoprecipitation [13, 14].

Because oocytes do not proliferate in culture and each zebrafish has a limited number of early-stage oocytes, techniques like those described above that require large amounts of tissue are not feasible. We decided to use a method that was as direct as possible to minimize the loss of material and takes advantage of the Balbiani body retaining its shape for long periods of time in simple culture media [15, 16]. Thus we anticipated and found that Balbiani bodies could be manually collected. This method is similar to how germ cells have been isolated from early Xenopus embryos [17]. The zebrafish is an excellent model organism for this method because its genome and transcriptome sequences are complete, the Balbiani body is large enough to isolate manually, and there are good genetic tools for future study of identified proteins. We use juvenile zebrafish because they have almost exclusively stage I oocytes, which contain Balbiani bodies, and few later stage oocytes [18].

We were able to collect enough material to successfully perform mass spectrometry and identify over 200 proteins present in the Balbiani body-enriched sample, including both previously known and novel components. We expect that this method could be used for RNAseq analysis to identify the mRNA cargo of the Balbiani body. The isolated Balbiani bodies can also be used for in vitro experiments and live imaging.

2 Materials

Prepare the following reagents and materials prior to beginning the Balbiani body isolation procedure. Follow IACUC guidelines for husbandry and euthanasia of zebrafish. Solutions are prepared at room temperature (RT; 22 °C) using ultrapure water unless indicated otherwise. Safely dispose of all glass and sharps.

1. Juvenile zebrafish, four to six weeks old (2 cm long from tip to base of tail).
2. Glass dish (5–10 cm diameter).
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, KH_2PO_4 1.8 mM, pH 7.4.
4. Two Dumont #55 forceps.
5. Vannas Spring Scissors.
6. L-15 Medium (Leibovitz).

7. Ovary digestion mix: 1 mg/mL Collagenase I, 1 mg/mL Collagenase II, 0.5 mg/mL hyaluronidase in L-15 Medium. Store all digestion enzymes at −20 °C as 100 mg/mL stock solutions. To 1 mL L-15 Medium, add 10 μL of Collagenase I stock solution, 10 μL of Collagenase II stock solution, and 5 μL hyaluronidase stock solution.
8. 100 μm Cell strainer.
9. 6-Well cell culture plate.
10. $DiOC_6$: 5 mg/mL in EtOH.
11. 1× Protease inhibitor cocktail in 10 mL PBS: Add 1 protease inhibitor tablet to 10 mL of PBS. Vortex vigorously until tablet is dissolved. Keep on ice. Stored protease inhibitor solution quickly loses its activity, so the protease inhibitor cocktail must be prepared immediately before the experiment.
12. 9-Well clear glass spot plate.
13. Dispenser syringe tips for repeating pipettor: 0.6 mL volume.
14. Needles: 30 gauge.
15. Microinjection needles: Use thin-wall borosilicate glass capillary tubes with filament: 1.0 mm outer diameter, 0.75 mm inner diameter, 4 in. long. Pull to a fine, long tip (approximately 10.5 mm taper).
16. Drummond Scientific Microcap Bulb Dispenser.

3 Methods

3.1 Isolating Stage I Oocytes

1. Euthanize juvenile fish in ice-cold water.
2. On a paper towel, cut behind the gills to remove the head of the fish. Then cut off the tail between the pelvic fin and the anal fin. Finally, while holding the torso of the fish in forceps, cut along the ventral midline of the fish (Fig. 1a).
3. Place the fish torso in a glass dish filled with approximately 5 mL of PBS.
4. Pull the ovaries out of the body cavity (*see* **Note 1**). The ovaries are anchored within the zebrafish to the swim bladder, the body wall, and fat deposits. Use forceps to dissect away any tissue stuck to the ovary (*see* **Note 2**) (Fig. 1b).
5. Add ovaries to a microfuge tube containing 1 mL of digestion mix. Pooling 5–8 ovaries will provide enough stage I oocytes to collect sufficient Balbiani bodies.
6. Incubate on a test tube rotator for 30 min at RT. Do not vortex or shake too hard: this will break up the larger oocytes and add debris to the isolated oocytes.

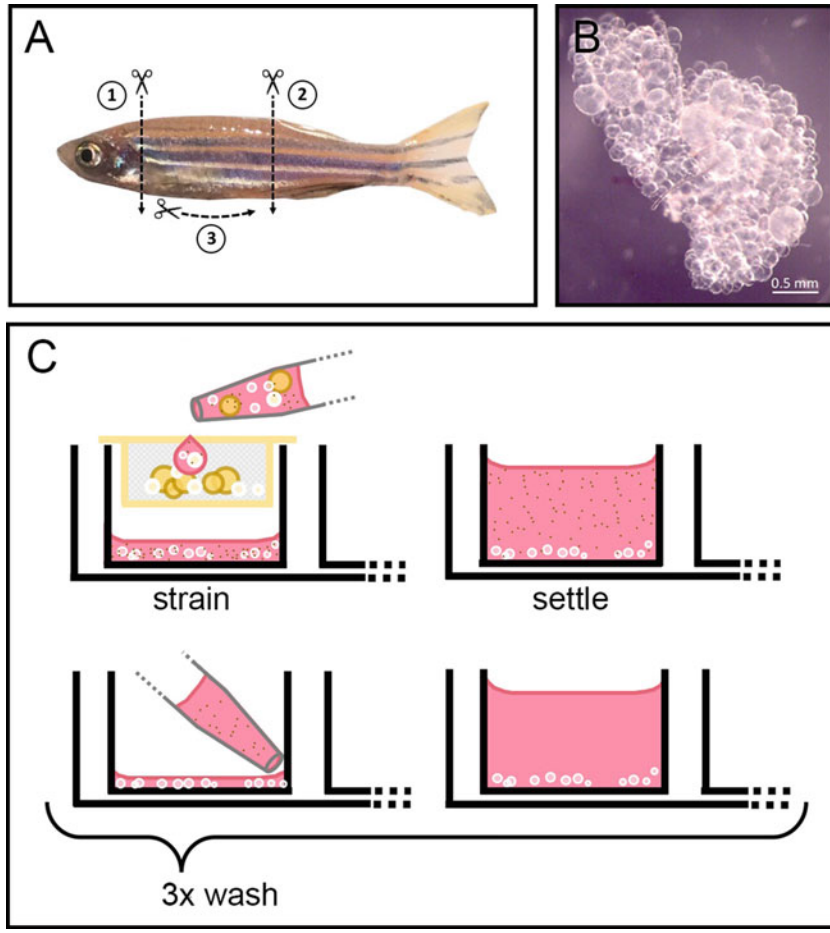

Fig. 1 Isolation of stage I oocytes from juvenile zebrafish. Panel (**a**) shows the three cuts used to access the ovary. (1) Vertically behind the gills to remove the head. (2) Vertically between the pelvic and anal fin to remove the tail. (3) Along the ventral midline to open the body cavity. Panel (**b**) shows a juvenile ovary with extra adhering tissue removed. Scale bar 0.5 mm. Panel (**c**) is a schematic of the washing process. The black lines represent one well of a 6-well plate. Strain: The digested ovary is passed through a 100 μm cell strainer to remove large oocytes. Settle: The oocytes that passed through the strainer settle to the bottom of the well. Wash: The media above the oocytes is removed and replaced three times to remove digestive enzymes and small debris (yolk particles and small oocytes)

7. Pass the digested ovary mix through a 100 μm cell strainer into one well of a 6-well culture plate (Fig. 1c, *see* **Note 3**). Because oocytes will lyse if they touch any dry surfaces, wet the mesh of the strainer with L-15 media before pouring oocytes over it.
8. Gently rinse the oocytes in the strainer with more L-15 media until only large unwanted oocytes are present in the strainer and the well is mostly filled.
9. To remove digestion enzymes and debris smaller than 100 μm, wash the oocytes multiple times with L-15 media. The stage I

oocytes will settle to the bottom of the well first, and the debris-containing media can be removed with a transfer pipette (Fig. 1c). Perform this step under a dissecting microscope to make sure that none of the desired oocytes are taken up, and to check that all the debris has been removed.

3.2 Collecting Balbiani Bodies

1. Add a 1:5000 dilution of the $DiOC_6$ stock solution to the oocytes for a final concentration of 1 μg/mL. The $DiOC_6$ does not mix well with water, so it is easier to first dilute 10 μL of $DiOC_6$ to 500 μL of L-15 media, and then add 10 μL of the diluted $DiOC_6$ for each 1 mL of L-15 media covering the oocytes to reach a 1:5000 dilution. The wells in a 6-well plate hold approximately 15 mL of media.
2. Let oocytes incubate with the dye for 1–2 h in a 28 °C incubator.
3. Add 30 μL of fresh protease inhibitor cocktail to a 0.5 mL microfuge tube, where collected Balbiani bodies will eventually be transferred. Keep on ice (*see* **Note 4**).
4. Under a fluorescent dissecting microscope, swirl the stained oocytes to bring them into the center of the well. With a pipettor, move 10 μL of oocytes to one well of the glass spot plate (*see* **Note 5**) (Fig. 2a).
5. Add 250 μL of fresh protease inhibitor cocktail to the oocytes.
6. Cut the tip off of the 0.6 mL syringe, removing about 1 cm. Fit a 30 gauge needle to the end (Fig. 2b). If it does not fit snugly, cut off more of the syringe tip. Move the syringe plunger up and down to make sure that it is not sticking.
7. Pass the oocytes through the needle 2–5 times, until there are only a few intact oocytes left. To minimize the air pulled into the needle, turn the beveled end down, flush against the glass, and keep it flat as you pass it over the oocytes (Fig. 2c).
8. The lysed oocyte mixture will initially be cloudy. Wait for 5–10 min for the Balbiani bodies and other cell parts to sink.
9. With forceps, break the tip of the microinjection needle to a diameter of 20–30 μm.
10. Using the microscope's green fluorescence channel under maximum zoom (6.3×), identify Balbiani bodies. Balbiani bodies will be about 10–30 μm in diameter and are the most intensely stained structures. To better identify Balbiani bodies, examine the edges. Balbiani bodies are usually not perfectly spherical, and have slightly fuzzy edges. They look like balls of lint or felt, not bubbles or glass beads. Any object with perfectly round, smooth edges is likely not a Balbiani body and is instead some other debris, such as a cortical granule or tiny unlysed oocyte (*see* **Note 6**) (Fig. 2d).

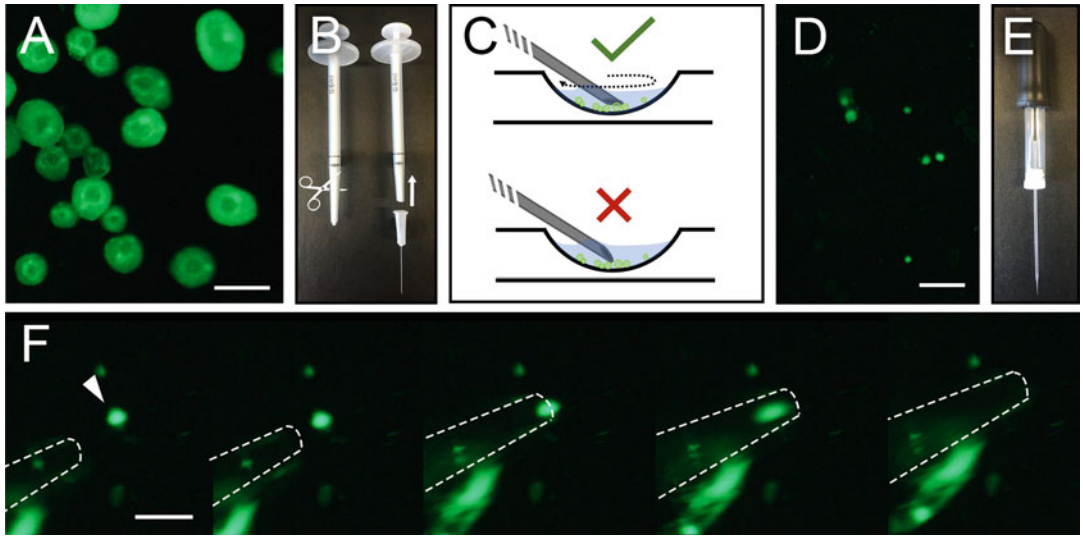

Fig. 2 Lysis of oocytes and collection of Balbiani bodies. Panel (**a**) shows whole stage I oocytes stained with $DiOC_6$ before lysis. The Balbiani body can be seen as a bright spot beside the nucleus in most oocytes. Panel (**b**) shows the preparation of the needle and syringe. Panel (**c**) shows the correct orientation of the beveled needle tip so that it points downward toward the bottom of the spot plate well. Panel (**d**) shows Balbiani bodies at the bottom of the well. To show the range of sizes, several dispersed Balbiani bodies have been moved into a single cluster. Panel (**e**) shows the bulb dispenser attached to the glass injection needle. Panel (**f**) is a series of consecutive frames from a video where a Balbiani body is drawn into the glass needle. The dotted line shows the outline of the needle. Note that the small debris around the Balbiani body is not taken up into the needle. Scale bars are 100 μm

11. Place the broken tip of the microinjection needle next to a Balbiani body. Capillary action should draw the Balbiani body up into the needle, along with a small amount of buffer. If the needle immediately clogs or if the Balbiani body cannot fit through, break a larger opening with forceps.
12. Move the tip of the needle from Balbiani body to Balbiani body, collecting several per needle (*see* **Note 7**) (Fig. 2f).
13. When the needle clogs or has drawn up 1–2 μL of liquid, transfer the Balbiani bodies to a tube. Attach the Microcap dispenser bulb to the open end of the needle (Fig. 2e). Carefully lower the tip of the needle into the 0.5 mL microfuge tube until it is just submerged in the protease inhibitor cocktail. Carefully dispense the Balbiani bodies into the tube (*see* **Note 8**).
14. After transferring Balbiani bodies, break the tip of a fresh needle and repeat the collection process (**step 11** onward).
15. When there are no more Balbiani bodies in the current well, transfer another 10 μL of oocytes to a fresh well in the glass spot plate and repeat lysis and collection (**step 5** onward).

16. Periodically, spin the Balbiani bodies down and remove the buffer that has collected in the tube. Too much buffer will cause the Balbiani bodies to float and stick to the sides of the microfuge tube, where they will be inaccessible for analysis.
17. Check the collected Balbiani bodies to count approximately how many have been collected. We found that 150–200 could produce good proteomics results, and we estimated that this represented between 0.5 and 1 μg of material.
18. Spin down the sample and remove as much buffer as possible.
19. Store Balbiani bodies at −80 °C until ready for analysis.

4 Notes

1. At this size, some of the fish will have converted their protogynous gonad to testes [18, 19]. Testes are mottled milky white, and more elongated than ovaries.
2. Ovaries that are suitable for Balbiani body isolation are small and clear. An opaque, yellow ovary contains late-stage oocytes that will add extra debris to the preparation. Juvenile fish that have been well fed may have ovaries that develop late-stage oocytes earlier. Conversely, older fish may have underdeveloped ovaries that are useable.
3. A 6-well plate is ideal because the cell strainer fits into it and the area at the bottom of the well is small. In a large, shallow dish it is harder to wash the oocytes. Also, the 6-well plate makes it easy to test two conditions in parallel.
4. Using a smaller tube makes the Balbiani bodies less likely to stick high up on the sides of the tube and become inaccessible for analysis. A 0.2 mL tube is also a good alternative.
5. Gently swirl the media to collect the oocytes at the center of the well to make them easier to transfer. The tip of an unbroken injection needle can be used to move Balbiani bodies around in the bottom of the glass well. The side of the unbroken needle can also be used like a knife to cut unlysed oocytes in half, freeing the Balbiani body.
6. Among the lysate will be oocyte nuclei. The $DiOC_6$ dye will stain the outer membrane of the nucleus and each nucleus will look like a bubble (dark in the middle). They are also slightly larger than the Balbiani bodies.
7. Some Balbiani bodies will stick to the sides of the glass needle and some loss has been unavoidable. Collecting the Balbiani bodies quickly helps reduce sticking.
8. To avoid crushing the needle into the bottom of the tube, it helps to lift the tube out of the ice and perform the transfer at

eye level. To make sure that all the liquid transfers, squeeze microcap dispenser bulb until bubbles come from the needle tip.

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Chapter 18

Generation of *Xenopus* Haploid, Triploid, and Hybrid Embryos

Romain Gibeaux and Rebecca Heald

Abstract

Frog species of the genus *Xenopus* are widely used for studies of cell and developmental biology, and recent genome sequencing has revealed interesting phylogenetic relationships. Here we describe methods to generate haploid, triploid, and hybrid species starting from eggs and sperm of *Xenopus laevis* and *Xenopus tropicalis* that enable investigation of how genome size and content affect physiology at the organismal, cellular, and subcellular levels.

Key words *Xenopus*, *Xenopus laevis*, *Xenopus tropicalis*, Fertilization, Embryology, Ploidy, Haploid, Triploid, Hybridization, Hybrid

1 Introduction

Although central to evolution, the biological consequences of changes in ploidy or genome hybridization are poorly understood. Amphibian genomes exist in a range of sizes and external fertilization in vitro has permitted manipulation of ploidy in various ways [1, 2]. *Xenopus* frog species are most widely studied and occupy a range of ploidies from diploid *Xenopus tropicalis* (2 N, 20 chromosomes) to dodecaploid *Xenopus longipes* (12 N, 108 chromosomes) [3]. Cross-fertilization between species is possible, as well as the generation of haploid and triploid embryos [4–10]. The most commonly used species, *Xenopus laevis*, is an allotetraploid with 36 chromosomes that arose through interspecific hybridization of diploid *X. tropicalis*-like progenitors that diverged from a common ancestor ~48 million years ago [11]. Interestingly, whereas the hybrid produced when *X. laevis* eggs are fertilized by *X. tropicalis* sperm ($l_e \times t_s$) is viable, the reverse hybrid ($t_e \times l_s$) dies prior to gastrulation [8, 9]. In this protocol, we describe simple and optimized methods to generate haploid, triploid, and hybrid embryos of the *Xenopus* species, *X. laevis* and *X. tropicalis* (Fig. 1). The protocols are organized according to maternal species. Sperm

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_18,

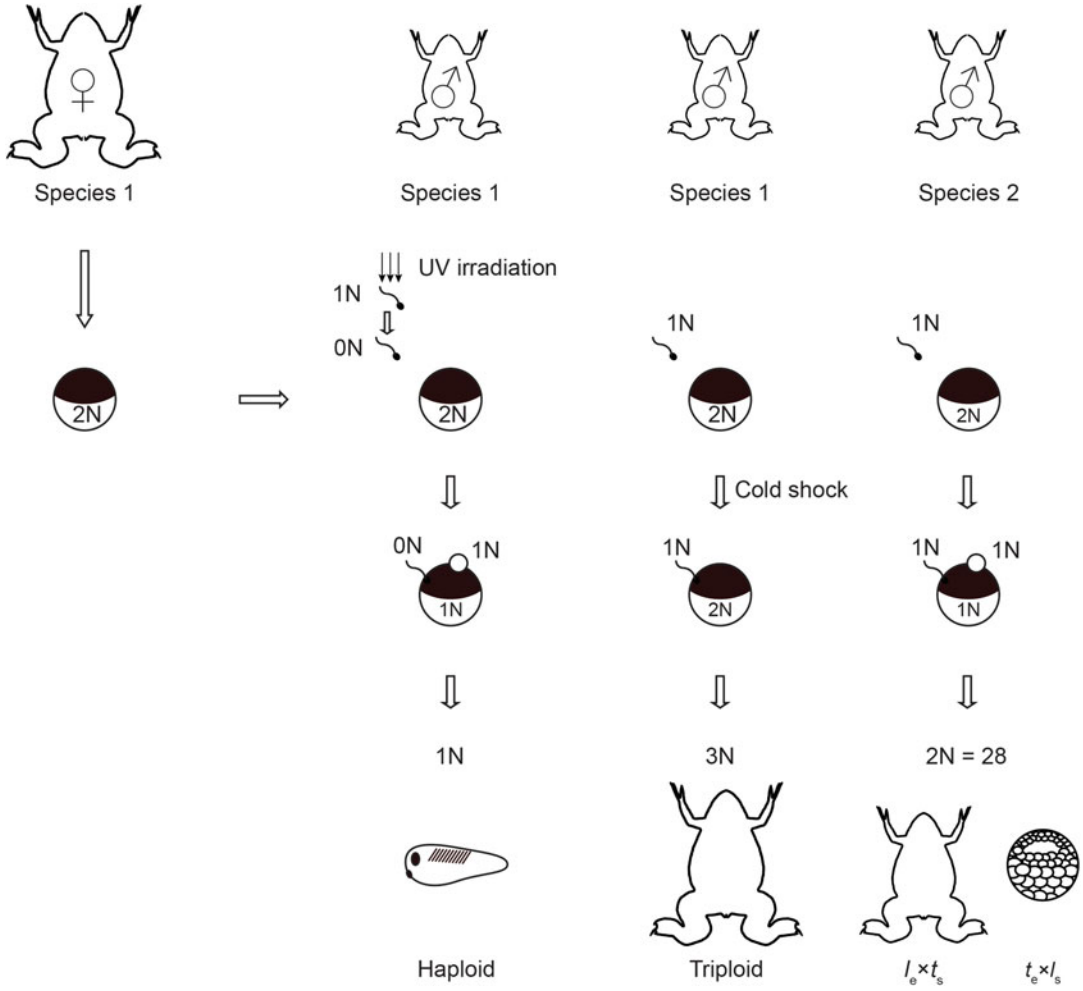

Fig. 1 Overview of ploidy manipulation in *Xenopus*. Irradiation of sperm is used to inactivate the paternal genome and generate haploid embryos. Haploid embryos develop as stunted tadpoles that never reach metamorphosis and exhibit the so-called haploid syndrome (*see* **Note 6**). Cold shock of fertilized eggs is used to block polar body extrusion and generate triploid embryos. Triploid embryos are viable and can develop to adult frogs (*see* **Note 8**). Fertilization of eggs with sperm of a different species produces hybrid embryos. Whereas the hybrid produced when *X. laevis* eggs are fertilized by *X. tropicalis* sperm ($l_e \times t_s$) is viable and develops to adult frog, the reverse hybrid ($t_e \times l_s$) dies prior to gastrulation

irradiation inactivates the paternal genome to generate haploids, while cold shock of eggs blocks polar body extrusion to generate triploid embryos. These are the simplest methods to perturb *Xenopus* embryo ploidy. Finally, we describe optimized methods to generate the viable hybrid obtained from the fertilization of *X. laevis* eggs with *X. tropicalis* sperm ($l_e \times t_s$) as well as the inviable hybrid obtained from the fertilization of *X. tropicalis* eggs with *X. laevis* sperm ($t_e \times l_s$), with reproducible fertilization efficiencies.

Thus, this protocol provides methods to prepare *Xenopus* embryos with varying genome sizes and content.

2 Materials

1. Pregnant mare serum gonadotropin (PMSG): 200 IU/mL PMSG in sterile Milli-Q H_2O. Store at 4 °C.
2. Human chorionic gonadotropin hormone (HCG): 1000 IU/mL HCG in sterile Milli-Q H_2O. Store at 4 °C.
3. 10× Modified Ringer's solution (MR): 1 M NaCl, 18 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$, and 50 mM HEPES-NaOH pH 7.6. Prepare 500 mL in deionized H_2O and filter sterilize. Store at room temperature.
4. 20× Marc's modified Ringer's solution (MMR): 2 M NaCl, 40 mM KCl, 40 mM $CaCl_2$, 20 mM $MgCl_2$, 100 mM HEPES (free acid), 2 mM EDTA, NaOH pH 7.8. Prepare 1 L in deionized H_2O and filter sterilize. Store at room temperature.
5. 1.5% Agarose in 1/10× MMR: Add 1.5 g agarose to 100 mL 1/10× MMR and microwave until dissolved. Melt in microwave before using.
6. Dejellying solution: 3% L-Cysteine in Milli-Q H_2O. Adjust to pH 7.8 with 10 N NaOH. Prepare 100 mL fresh just before use.
7. 10% of fetal bovine serum in Leibovitz's L-15 Medium: Leibovitz's L-15 Medium is stored at 4 °C. FBS is stored at −20 °C as 500 μL aliquots. Prepare 1.1 mL of 10% FBS in Leibovitz's L-15 Medium fresh just before use.
8. Plastic containers for frogs (4 L for *X. laevis* and 6 L for *X. tropicalis*) with tight-fitting lids and holes punched for air exchange.
9. Room (16 °C) or large non-airtight incubator set to 16 °C for *X. laevis*.
10. 1 mL Syringes.
11. 30 gauge needles.
12. Forceps.
13. Dissection scissors.
14. Plastic pestle for 1.5 mL microfuge tube.
15. Petri dishes (glass, 6 cm diameter, with lids).
16. Plastic squeeze bottles.
17. Transfer pipets.
18. Dissection stereomicroscope.

19. Stratalinker UV-Crosslinker (Stratagene, San Diego, CA).
20. Rectangular ice bucket.
21. Rectangular plastic dish (~12 × 9 × 4 cm) with walls as thin as possible (e.g., pipette tip box lid).

3 Methods

3.1 Methods based on X. laevis Eggs

3.1.1 Preparation for X. laevis Egg Fertilization

1. Prime *X. laevis* females by injecting 0.5 mL (100 IU) of PMSG subcutaneously into the dorsal lymph sac using a 30 gauge needle and 1 mL syringe, at least 48 h before boosting (*see* **Note 1**).
2. As needed, prime one *X. laevis* male by injecting 0.5 mL (500 IU) of HCG or one *X. tropicalis* male by injecting 0.25 mL (250 IU) of HCG, about 24 h before dissection.
3. Carry out **steps 3–6** the day before fertilization. Boost *X. laevis* females by injecting 0.5 mL (500 IU) of HCG to induce ovulation (*see* **Note 2**). Store each frog individually in 2 L of freshly prepared 1× MMR in a 4 L plastic container overnight at 16 °C.
4. If applicable: Euthanize the *X. laevis* male and dissect to extract the testes. Clean testes free of fat or blood vessels, and store at 4 °C in a glass petri dish filled with 1× MR (*see* **Note 3**).
5. Prepare fresh 0.5–1 L of 1/10× MMR to be used for the preparation of petri dishes and fertilization.
6. Prepare fertilization petri dishes by coating the bottom with 5–10 mL melted 1.5% agarose in 1/10× MMR. Once solidified, cover the agarose with 1/10× MMR, and store the dishes at room temperature to prevent them from drying out.
7. Carry out **steps 7–9** on the day of fertilization. Analyze egg quality and select the females to be used. Avoid females that have laid lysed or stringy eggs (*see* **Note 4**).
8. Fill a plastic squeeze bottle with 1/10× MMR.
9. Discard the 1/10× MMR covering the agarose from the fertilization petri dishes.

3.1.2 Generation of Haploid X. laevis

1. Cut 1/2–2/3 of a testis using forceps and scissors and add it to 1.1 mL of Milli-Q H_2O in a 1.5 mL microcentrifuge tube.
2. For selected *X. laevis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization petri dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).

3. Gently swirl the dish to form a monolayer of eggs.
4. Prepare sperm solution by cutting the piece of testis into smaller, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle.
5. Spin down briefly using a benchtop centrifuge to pellet the tissues.
6. Collect 1 mL of supernatant, without pipetting any pieces of tissue, and transfer into a non-agarose-coated glass petri dish. Swirl to distribute the solution evenly.
7. Place the open dish into the UV-Crosslinker and irradiate the solution two times with 30,000 microjoules, swirling the solution between the two irradiations (*see* **Note 6**).
8. Retrieve the irradiated sperm and fertilize by depositing 0.5 mL of irradiated sperm solution on top of the eggs.
9. Use a clean plastic pestle to gently mix the eggs within the sperm solution and incubate for 10 min (*see* **Note 7**). Keep the dish slanted during this step to maximize the interaction of the sperm with the eggs.
10. Flood the fertilization dish with 1/10× MMR, making sure to submerge all eggs, and incubate for 10 min.
11. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
12. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.1.3 Generation of Triploid X. laevis

1. Fill a rectangular ice bucket with slushy ice, and partially submerge a rectangular plastic dish and a 50 mL conical tube filled with 1/10× MMR (Fig. 2). Store at 4 °C for at least 2 h.
2. Prepare cold shock-ready fertilization petri dishes by coating the bottom with only ~1.5 mL of melted 1.5% agarose in 1/10× MMR, 1–2 h ahead.
3. Cut 1/3–1/2 of a testis using forceps and scissors and add it to 1 mL of Milli-Q H_2O in a 1.5 mL microcentrifuge tube.
4. For selected *X. laevis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
5. Gently swirl the dish to form a monolayer of eggs.

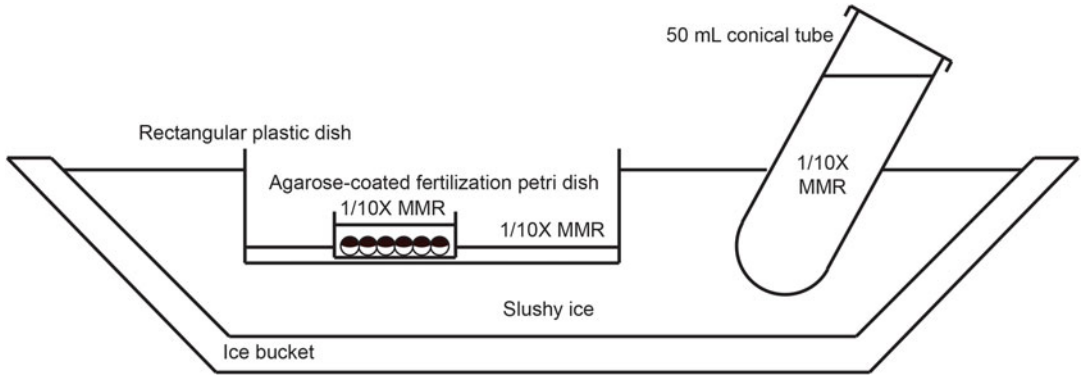

Fig. 2 Cold-shock treatment apparatus. The rectangular ice bucket is filled with slushy ice. A rectangular plastic dish and a 50 mL conical tube filled with 1/10× MMR are partially submerged and stored at 4 °C for at least 2 h. To proceed with the cold shock, the fertilization petri dish (coated with 1.5 mL of 1.5% agarose in 1/10× MMR) containing the fertilized eggs and ice-cold 1/10× MMR is placed, without its lid, in the cooled plastic dish, and the rest of the ice-cold 1/10× MMR is used to fill the cooled plastic dish

6. Prepare sperm solution by cutting the piece of testis into smaller, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle.
7. Fertilize the eggs by transferring the 1 mL of sperm solution on top of the eggs.
8. Use a clean plastic pestle to gently mix the eggs within the sperm solution. Make sure that the eggs make a monolayer and incubate for 10 min (*see* **Note 7**).
9. Flood the fertilization dish with 1/10× MMR, making sure to submerge all eggs, and incubate for 3 min.
10. Exactly 13 min after sperm addition, rapidly remove the 1/10× MMR using a plastic transfer pipette, pour ice-cold 1/10 MMR from the 50 mL conical tube, place the fertilization petri dish in the cooled plastic dish, and add the rest of the ice-cold 1/10× MMR to the cooled plastic dish (Fig. 2). Incubate for 15 min. Leave the lid of the fertilization petri dish at room temperature (*see* **Note 8**).
11. Remove the petri dish from the plastic dish, and exchange the cold 1/10× MMR for room-temperature 1/10× MMR. Close the fertilization petri dish with its lid left at room temperature, and let the temperature equilibrate on the bench for 25–30 min.
12. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.

13. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.1.4 Generation of $|_e \times t_s$ Hybrids

1. Euthanize the *X. tropicalis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and store at room temperature in 1 mL of L15 supplemented with 10% FBS until they are used for fertilization (*see* **Note 9**).
2. For selected *X. laevis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
3. Gently swirl the dish to form a monolayer of eggs.
4. Transfer one testis in 1 mL of Milli-Q H_2O and prepare the sperm solution by cutting the testis into small, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle.
5. Fertilize by depositing 1 mL of sperm solution on top of the eggs (*see* **Note 10**).
6. Use a clean plastic pestle to gently mix the eggs within the sperm solution, and incubate for 10 min (*see* **Note 7**). Keep the dish slanted during this step to maximize the interaction of the sperm with the eggs.
7. Flood the fertilization dish with 1/10× MMR, making sure to submerge all eggs, and incubate for 10 min.
8. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
9. Remove the dejellying solution and wash 3–5 times with 1/10X MMR.

3.2 Methods based on X. tropicalis Eggs

3.2.1 Preparation for X. tropicalis Egg Fertilization

1. Carry out **steps 1–5** the day before fertilization. As applicable: Prime one *X. laevis* male by injecting 0.5 mL (500 IU) of HCG or one *X. tropicalis* male by injecting 0.25 mL (250 IU) of HCG, about 24 h before dissection.
2. Prime *X. tropicalis* females by injecting 0.25 mL (25 IU) of HCG subcutaneously into the dorsal lymph sac using a 30 gauge needle and 1 mL syringe, 20 h before boosting (*see* **Note 11**).
3. If applicable: Euthanize the *X. laevis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and store at 4 °C in a glass petri dish filled with 1× MR (*see* **Note 12**).

4. Prepare fresh 0.5–1 L of 1/10× MMR to be used for the preparation of the dishes and fertilization.
5. Prepare fertilization dishes by coating their bottom with 5–10 mL melted 1.5% agarose in 1/10× MMR. Once solidified, cover the agarose with 1/10× MMR, and store the dishes at room temperature.
6. Carry out **steps 6–9** on the day of fertilization. Boost *X. tropicalis* females by injecting 0.25 mL (250 IU) of HCG to induce ovulation. Store each frog individually in 2–3 L of deionized H_2O in a 6 L plastic container at room temperature.
7. Analyze egg quality and select the females to be used. Avoid females that have laid lysing or stringy eggs (*see* **Note 4**). Always use the frogs shortly after first eggs are laid (*see* **Note 13**).
8. Prepare plastic squeeze bottles filled with Milli-Q H_2O and 1/10× MMR.
9. Discard the 1/10× MMR covering the agarose from the fertilization petri dishes.

3.2.2 Generation of Haploid X. tropicalis

1. Euthanize the *X. tropicalis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and place in 1.1 mL of L15 supplemented with 10% FBS (*see* **Note 9**).
2. For selected *X. tropicalis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization petri dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
3. Gently swirl the dish to form a monolayer of eggs.
4. Prepare sperm solution by cutting the testes into small, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle within the L15 + 10% FBS.
5. Spin down briefly using a benchtop centrifuge to pellet the tissues.
6. Collect 1 mL of supernatant, without pipetting any pieces of tissue, and transfer into a non-agarose-coated glass petri dish. Swirl to distribute the solution evenly.
7. Place the open dish in the UV-Crosslinker, and irradiate the solution once with 50,000 microjoules (*see* **Note 6**).
8. Retrieve the irradiated sperm and fertilize by depositing 0.5 mL of irradiated sperm solution on top of the eggs.
9. Use a clean plastic pestle to gently mix the eggs within the sperm solution, and incubate for 4–5 min (*see* **Note 7**). Keep

the dish slanted during this step to maximize the interaction of the sperm with the eggs.

10. Flood the fertilization dish with Milli-Q H_2O, making sure to submerge all eggs, and incubate for 10 min.
11. Exchange the Mili-Q H_2O for 1/10× MMR, and incubate for 10 min.
12. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
13. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.2.3 Generation of Triploid *X. tropicalis*

1. Fill a rectangular ice bucket with slushy ice, partially submerge a rectangular plastic dish and a 50 mL conical tube filled with 1/10× MMR (Fig. 2). Store at 4 °C for at least 2 h.
2. Prepare cold shock-ready fertilization petri dishes by coating the bottom with only ~1.5 mL of melted 1.5% agarose in 1/10× MMR, 1–2 h ahead.
3. Euthanize the *X. tropicalis* male and dissect to extract the testes. Clean testes free of fat and blood vessels, and place both in 1 mL of L15 supplemented with 10% FBS (*see* **Note 9**).
4. For selected *X. tropicalis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
5. Gently swirl the dish to form a monolayer of eggs.
6. Prepare sperm solution by cutting the testes into small, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle within the L15 + 10% FBS.
7. Fertilize the eggs by transferring 0.5 mL of sperm solution on top of the eggs.
8. Use a clean plastic pestle to gently mix the eggs within the sperm solution. Make sure that the eggs form a monolayer and incubate for 4 min (*see* **Note 7**).
9. Flood the fertilization dish with Milli-Q H_2O, making sure to submerge all eggs, and incubate for 5 min.
10. Exactly 9 min after sperm addition, rapidly remove the Milli-Q H_2O using a plastic transfer pipette and pour ice-cold 1/10 MMR from the 50 mL conical tube, place the petri dish in the cooled plastic dish, and add the rest of the ice-cold 1/10 MMR

to the cooled plastic dish (Fig. 2). Incubate for 10 min. Leave the lid of the fertilization petri dish at room temperature (*see* **Note 8**).

11. Remove the fertilization dish from the plastic dish, and exchange the cold 1/10× MMR for room-temperature 1/10× MMR. Close the fertilization petri dish with its lid left at room temperature, and let the temperature equilibrate on the bench for 20 min.
12. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.
13. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.2.4 Generation of $t_e \times l_s$ Hybrids

1. Cut 1/2 of a *X. laevis* testis using forceps and scissors, and place it in 0.5 mL of L15 + 10% FBS in a 1.5 mL microcentrifuge tube.
2. For selected *X. tropicalis* females, promote egg-laying by gently squeezing females in a manner that mimics amplexus, and collect freshly laid eggs atop a fertilization dish. Obtain all necessary eggs from one squeeze. Remove any liquid that has dripped off of the frog during squeezing from the fertilization dish (*see* **Note 5**).
3. Gently swirl the dish to form a monolayer of eggs.
4. Prepare sperm solution by cutting the piece of testis into smaller, millimeter-sized pieces and homogenizing the solution using a clean plastic pestle within the L15 + 10% FBS.
5. Fertilize by depositing 0.5 mL of sperm solution on top of the eggs (*see* **Note 10**).
6. Use a clean plastic pestle to gently mix the eggs within the sperm solution and incubate for 5 min (*see* **Note 7**). Keep the dish slanted during this step to maximize the interaction of the sperm with the eggs.
7. Flood the fertilization dish with Milli-Q H_2O, making sure to submerge all eggs, and incubate for 10 min.
8. Exchange the Milli-Q H_2O for 1/10× MMR, and incubate for 10 min.
9. Exchange the 1/10× MMR for freshly prepared dejellying solution. Swirl gently and exchange for fresh dejellying solution 2–3 times, until embryos pack tightly together when the dish is tilted. Do not keep the eggs in the dejellying solution for more than a total of 10 min.

10. Remove the dejellying solution and wash 3–5 times with 1/10× MMR.

3.3 General Postfertilization Embryo Care

1. Let the embryos develop at 23 °C to stages 2–3 (*see* **Note 14**). Observe the embryos under a dissection stereomicroscope and sort them to remove unfertilized eggs and dying embryos using plastic transfer pipettes. As soon as the sorting is complete, transfer healthy embryos to fresh 1/10× MMR in a fresh fertilization dish in order to keep the embryos in a dish that has not been exposed to dejellying solution.
2. Let the embryos develop at 23 °C until the desired developmental stage (*see* **Note 15**). Observe the embryos 2–3 times a day and remove dying ones as soon as possible. Make sure to exchange for fresh 1/10× MMR every 10–14 h.

4 Notes

1. Priming the *X. laevis* female frogs increases egg yield and quality. Primed frogs should be used within two weeks.
2. *X. laevis* female boosting is usually performed the evening preceding the experiment, 16–18 h before eggs are needed.
3. Properly stored *X. laevis* testes can be used up to two weeks after dissection, but fertilization efficiency may decrease over time.
4. Egg quality is the primary and most important parameter as this will determine the efficiency of the fertilization. Careful analysis of egg quality is thus highly recommended.
5. Skin secretions from the frog will cause eggs to lyse. It is thus important to remove any liquid that has dripped off of the frog during squeezing from the fertilization dish.
6. The sperm irradiation method to produce *Xenopus* haploids is derived from experiments originally performed with *X. laevis* [4]. However, haploids can also be produced by irradiation of the eggs rather than the sperm [5]. Haploid embryos develop as stunted tadpoles that never reach metamorphosis. They exhibit the so-called haploid syndrome, which can be recognized easily by a shortened body axis, microcephaly, poorly formed gut, and edema [12, 13]. Note that the irradiation of sperm is the key step in the generation of haploids and, although we found the presented irradiation doses to be the most efficient, one can tweak this parameter if necessary.
7. Due to the robustness of wild-type fertilizations, control fertilizations with nonirradiated sperm (for haploid experiments), non-cold-shock-treated zygotes (for triploid experiments), or sperm from the same species (for hybrid experiments) can be

conducted with the same incubation procedures and timings as the ones optimized for the corresponding experiments with good fertilization efficiencies.

8. The cold-shock method to produce triploid embryos is derived from experiments originally performed with *X. laevis* [6]. Alternatively, triploids can be produced by exposing the zygote to hydrostatic pressure [7]. Triploid embryos are viable and can develop normally beyond metamorphosis [6]. Note that the key parameters for the success of the cold-shock treatment are the starting time (number of minutes postfertilization) and the duration of the shock, which can be adjusted if necessary.
9. *X. tropicalis* testes are always prepared immediately before use to ensure the highest fertilization efficiency possible.
10. Cross-fertilizations have different efficiencies. Fertilization efficiency of the viable $l_e \times t_s$ hybrid is high (close to 100%), while the $t_e \times l_s$ fertilization is much less efficient. With the optimized protocol presented here, we could occasionally reach 60–70% efficiency but, on average, one might expect only 10% efficiency.
11. Priming the *X. tropicalis* female frogs increases egg yield, as well as reproducibility in timing of laying.
12. Although properly stored *X. laevis* testes can be used up to two weeks, recently extracted testes (1–3 days) are preferred for cross-fertilization.
13. With the recommended hormone dosage and injection timing, ovulation usually occurs 3–3.5 h after boosting.
14. Embryos are staged according to Nieuwkoop and Faber [14].
15. Confirmation of changes in ploidy can be assessed in various ways, including counting the number of nucleoli, karyotyping, or genotyping if one uses parental frogs of different strains. In addition, by analyzing the size of the nucleus relative to the size of the cell using immunofluorescence, it is possible to discriminate the different ploidy of embryos starting from late stage 9 to stage 10 [15].

Acknowledgments

We thank members of the Heald lab for input. We thank Maiko Kitaoka and Kelly Miller for critical reading of the manuscript. RG was supported by an HFSP long-term fellowship LT 0004252014-L. RH was supported by NIH R35 GM118183 and the Flora Lamson Hewlett Chair.

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Chapter 19

Germ Cell Transplantation in Avian Species

Young Hyun Park, Young Min Kim, and Jae Yong Han

Abstract

Germ cell transplantation technology has played a critical role in germline modification and preservation of genetic resources. Several germ cell transplantation systems have been developed, including sperm, oocyte, or germline stem cell transplantation systems in mammals. Meanwhile, in avian species, this has mostly relied on primordial germ cell (PGC) transplantation for efficient germline transmission. In this chapter, we describe how to isolate PGCs from avian embryos and produce germline chimeras through transplantation of donor PGCs to recipient embryos.

Key words Aves, Germline chimera, Primordial germ cells, Transplantation

1 Introduction

Avian species are important not only as food resources but also for use in multidisciplinary studies across many fields including developmental biology, immunology, toxicology, physiology, and behavioral science [1, 2]. Therefore, the preservation of avian species is important for future generations [3]. However, unlike other model animals, it is difficult to perform technical procedures such as pro-nucleus injection into the oocyte, somatic cell nuclear transfer, embryonic stem cell (ESC)-mediated germline transmission, transgenesis, and genome editing in avian species due to their oviparous development and the physiological features of the ovum [4]. To overcome this limitation, much effort has focused on the development of a unique germ cell transplantation system via primordial germ cells (PGCs), which are progenitor cells of gametes containing genetic information that can be delivered to the next generation. Avian PGCs reportedly originate from maternally inherited germplasm factors, such as the chicken vasa homolog (*CVH*) and the chicken deleted in azoospermia-like (*DAZL*) gene, which indicates that avian germ cell specification follows the germplasm model [5, 6]. In the Eyal-Giladi and Kochav (EGK) stage X embryo, PGCs migrate toward the germinal crescent region until

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_19,

Hamburger and Hamilton (HH) stage 4 [7]. Subsequently, PGCs enter the vascular system via the anterior vitelline vein during HH stages 10–12, and they ultimately start to settle in the genital ridge at around HH stages 15–17 [8, 9]. Because avian PGCs have a unique migratory pathway during embryonic development, it is possible to isolate them from donor embryos and transplant them into recipient embryos [10–12]. For this reason, the PGC-mediated germ cell transplantation has been considered the most efficient germline transmission system in avian species, and it has successfully produced germline chimera and germline-transmitted progenies as well as transgenic and genome-edited progenies with much higher efficiencies than other germline-competent cells, such as ESCs and spermatogonial stem cells (SSCs) [13–16]. In this chapter, we introduce the methods to isolate vascular circulating PGCs using cell surface-specific antibody-mediated cell sorting and size-dependent cell isolation system, and describe the PGC-mediated transplantation system in detail.

2 Materials

2.1 Isolation and Purification of PGCs

2.1.1 Blood Isolation from Dorsal Aorta of HH Stage 13–16 Embryos

1. Korean Oge (KO) and white Leghorn (WL) chicken (*Gallus gallus domesticus*), Japanese quail (*Coturnix japonica*), mallard duck (*Anas platyrhynchos*), and Muscovy duck (*Cairina moschata*) at HH stage 13–16 [17] (*see* **Note 1**).
2. Microelectrode pipette puller.
3. Microgrinder.
4. 25 μm diameter glass micropipette.
5. Mouth-controlled pipette.
6. Sanitary cotton.
7. 70% Ethanol in distilled water.
8. 3.8% Sodium citrate buffer: 3.8% (w/v) Sodium citrate in distilled water, pH 7.2.
9. Sharpened microdissection forceps.
10. 1.5 mL Microtube.

2.1.2 PGC Purification Via Size-Dependent Isolation (SDI) System

1. 6.5 mm Transwell insert with 8 μm microporous membranes (Corning, CLS3422).
2. 1× Phosphate-buffered saline (1× PBS): 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic, and 1.8 mM monobasic potassium phosphate in distilled water, pH 7.2.
3. 3.8% Sodium citrate buffer.

4. Laboratory wipes (Kimberly-Clark Corp.).
5. Chicken DAZL (cDAZL) polyclonal antibody for purified PGCs (*see* **Note 2**).

2.1.3 PGC Purification Via Magnetic Activated Cell Sorting (MACS)

1. Magnetic activated cell sorting (MACS) separator (Miltenyi Biotec).
2. MACS column (MS columns, Miltenyi Biotec).
3. MACS buffer: 0.5% Bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA) in 1× PBS, pH 7.2.
4. Chicken PGC-specific surface antibody: Stage-specific embryonic antigen-1 (SSEA-1) antibody (Santa Cruz Biotechnology, Inc., SC-21702).
5. Anti-Mouse IgM MicroBeads (Miltenyi Biotec).
6. 1× PBS.

2.1.4 Immunocytochemistry of Purified PGC

1. Fixation solution: 4% (w/v) paraformaldehyde (Sigma-Aldrich) in 1× PBS.
2. Permeabilization solution: 0.1% Triton X-100 (Sigma-Aldrich) in 1× PBS.
3. Blocking solution: 10% (v/v) normal goat serum, 1% (w/v) BSA in 1× PBS.
4. Primary antibodies: SSEA-1 (Santa Cruz Biotechnology, Inc.).
5. Secondary antibodies: Goat anti-mouse IgM-phycoerythrin (PE) or mouse anti-rabbit IgG-fluorescein isothiocyanate (FITC).
6. ProLong Gold antifade reagent (with DAPI, or 4′,6-diamidino-2-phenylindole; Invitrogen).

2.2 PGC Transplantation

2.2.1 Fluorescent Labeling of Donor PGCs and Microinjection of PGCs into Recipient Embryos

1. Purified PGCs.
2. Fluorescent labeling dye: PKH26 red fluorescent cell linker kit (Sigma-Aldrich).
3. PKH26 staining stop solution: 10% (v/v) fetal bovine serum (FBS) in Dulbecco's modified Eagle medium (DMEM).
4. Hanks' balanced salt solution (HBSS).
5. Microelectrode pipette puller.
6. Microgrinder.
7. 25 μm diameter glass micropipette.
8. Mouth-controlled pipette.
9. Sharpened forceps for egg shell opening.
10. Parafilm.
11. HH stage 13–16 recipient chicken embryo.

2.2.2 Migration Assay of PGC

1. PKH26-labeled donor PGC-transplanted 6-day-old embryonic gonads (at HH stages 26–28).
2. Sterilized dissection petri dish.
3. Dissection pin.
4. Fine tweezers.
5. 1× PBS.
6. Inverted fluorescence microscope.

2.3 Test Cross and Identification of Germline Chimera

1. Sexually maturated male recipient (putative male germline chimera).
2. 1 mL syringe without needle (for artificial insemination).
3. 1.5 mL Microfuge tubes.
4. 1× PBS.
5. DNeasy Blood and Tissue Kit (QIAGEN).
6. Breed-specific primers for determination of KO chicken donor and WL chicken recipients [18]:

   KO-specific primers:

   – F: 5′-AGCAGCGGCGATGAGCAGCA-3′.
   – R: 5′-CTGCCTCAACGTCTCGTTGGC-3′.

   WL-specific primers:

   – F: 5′-AGCAGCGGCGATGAGCGGTG-3′.
   – R: 5′-CTGCCTCAACGTCTCGTTGGC-3′.
7. PCR kit (Takara, Ex Taq) or equivalent PCR kit.
8. PCR machine.

3 Methods

Here, we explain how to isolate avian PGCs from embryonic blood vessels using magnetic activated cell sorting (MACS) and size-dependent isolation (SDI), and describe production of germline chimeras in details (Fig. 1).

3.1 Isolation and Purification of PGCs

3.1.1 Isolation of Whole-Blood Cells from Avian Embryos

1. Incubate fresh eggs (EGK stage X) from various avian species such as white Leghorn chicken, Japanese quail, mallard duck, and Muscovy duck at 37 °C until the desired stage (HH stages 13–16) is reached. Incubate the fertilized mallard and Muscovy duck eggs until their morphology is similar to that of chicken or quail at the various stages (Fig. 2a).
2. After incubation, place the eggs horizontally on an egg plate and wipe the eggshells gently using sanitary cotton with 70% ethanol.

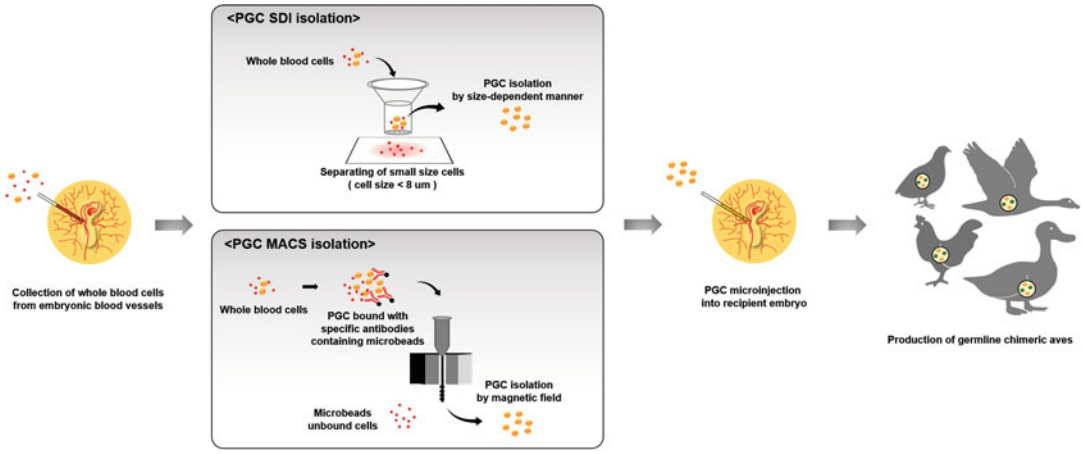

Fig. 1 Schematic representation of PGC isolation from avian embryonic blood vessels and subsequent transplantation. PGCs are isolated from avian embryonic blood vessels by size-dependent isolation (SDI) and magnetic activated cell sorting (MACS) system. Purified PGCs are injected into Hamburger and Hamilton (HH) stage 13–16 recipient embryos. Subsequently, the recipient embryos are hatched and raised until sexual maturation

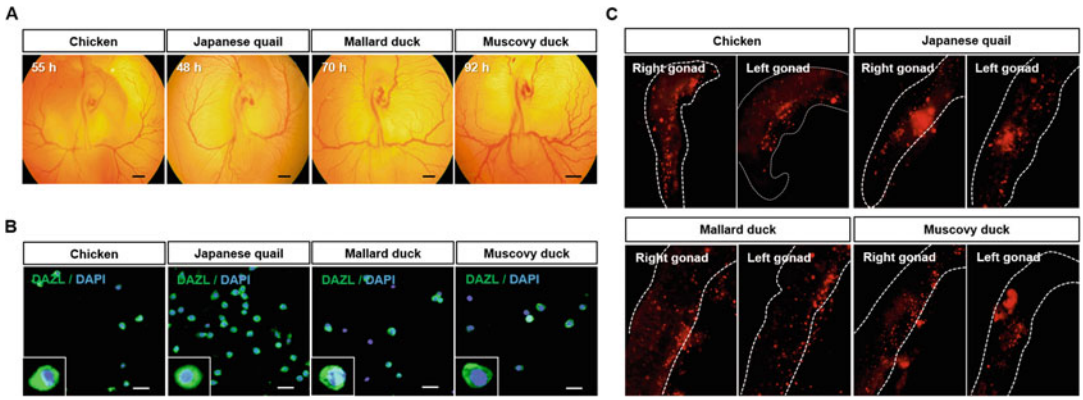

Fig. 2 Separation of avian PGCs by SDI system. (**a**) HH stage 13–16 embryos of the various avian species. Fertilized eggs of white Leghorn chickens, Japanese quail, mallard ducks, and Muscovy ducks are incubated for 55, 48, 70, and 92 h, respectively. Scale bar, 1 mm. (**b**) Immunocytochemical analysis of SDI-purified cells against DAZL in the various avian species. Scale bar, 50 μm. (**c**) Migration of SDI-purified cells in recipient chicken embryos. Approximately 500 cells isolated from the four avian species are labeled with PKH26 fluorescent dye, injected into the dorsal aorta of HH stage 13–16 chicken embryos, and incubated until HH stage 27 (reproduced from ref. [17] with permission from *Molecular Reproduction and Development*)

3. Using sharpened forceps, cautiously crack the eggshell (<1 cm diameter for the chicken and ducks, and <0.5 cm diameter for the quail) to isolate whole-blood cells.
4. Collect approximately 2–5 μL of embryonic blood from the dorsal aorta of each embryo using a thinly ground glass micropipette and mouth pipette under a microscope.

5. Transfer whole-blood cells to 1.5 mL microtubes with 100 μL of 3.8% sodium citrate buffer to prevent blood coagulation, and then centrifuge the whole blood cells at 200 × *g* for 5 min for further isolation of PGCs.

3.1.2 PGC Isolation Via SDI System

1. For PGC isolation via SDI system (Fig. 1), rinse a 6.5 mm Transwell insert with 8 μm microporous membranes with 500 μL of 1× PBS.
2. Resuspend the isolated embryonic blood cells in 500 μL of 3.8% sodium citrate buffer, pipette onto the Transwell insert, allow to pass through, and wash twice with 1× PBS using laboratory wipes.
3. Resuspend the cells on the microporous membranes in 500 μL of 1× PBS (*see* **Note 3**).
4. Centrifuge the suspended cells at 200 × *g* for 5 min and then resuspend the pellets in 1× PBS.
5. Confirm the identity of these cells by immunocytochemistry using cDAZL polyclonal antibody (Fig. 2b).

3.1.3 PGC Isolation Via MACS System

1. For PGC isolation via MACS system (Fig. 1), resuspend the cell pellets and label with anti-SSEA-1 antibody for chicken at 1:200 titers in 1 mL PBS; then incubate the cells for 15 min at room temperature (RT).
2. Wash the cells to remove unbound primary antibody by adding 5 mL MACS buffer per 10^7 total cells and centrifuge at 200 × *g* for 5 min.
3. Aspirate supernatant completely and resuspend cell pellet in 80 μL MACS buffer per 10^7 total cells.
4. Add 20 μL rat anti-mouse IgM MicroBeads per 10^7 cells. For higher cell numbers, scale up the buffer volume accordingly.
5. Incubate the cells for 20 min at 2–8 °C.
6. Wash the cells by adding 2 mL MACS buffer per 10^7 cells and centrifuge at 200 × *g* for 5 min.
7. Aspirate supernatant completely and resuspend up to 10^7 cells in 500 μL MACS buffer.
8. Place MACS column in the magnetic field of a suitable MACS separator.
9. Prepare column by rinsing with 500 μL MACS buffer.
10. Apply cell suspension to the column. Wash the column with 500 μL of MACS buffer three times and collect unlabeled cells that pass through. New buffer should be added only when the column reservoir is empty.
11. Remove the column from the separator and place it on a suitable collection tube.

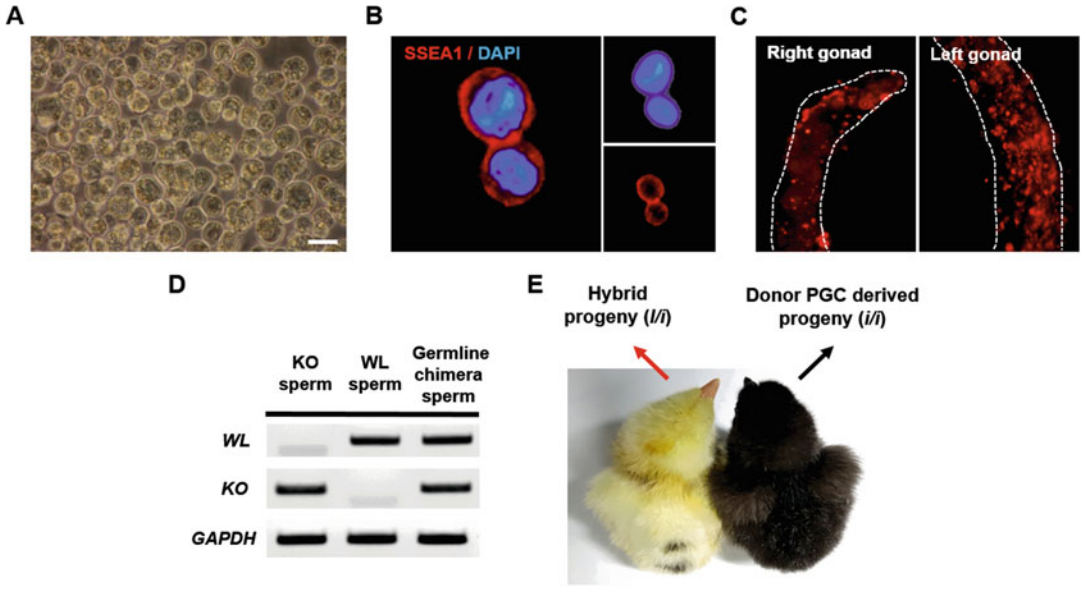

Fig. 3 Separation of avian PGCs by MACS and generation of donor PGC-derived progeny via PGC transplantation. (**a**) PGCs are isolated by MACS from HH stage 13–16 Korean Oge (KO) embryonic blood. Scale bar, 20 μm. (**b**) Immunocytochemical analysis of MACS-purified cells against SSEA1 in KO chicken embryonic blood is performed. (**c**) Approximately 500 cells isolated from KO chicken blood vessels are labeled with PKH26 fluorescent dye, injected into the dorsal aorta of HH stage 13–16 WL chicken embryos, and incubated until HH stage 27. (**d**) Genomic DNA PCR for breed determination of germline chimeric rooster. Genomic DNA is isolated from germline chimera sperm and analyzed by PCR using WL and KO chicken-specific primers. (**e**) Donor KO (*i/i*) PGCs are injected into the dorsal aorta of WL (*I/I*) recipient embryos, and after sexual maturation progeny is derived from the donor KO PGCs (black arrow, *i/i*). Hybrid progeny (red arrow, *I/i*) derived from the endogenous WL PGCs exhibits slightly yellow feathers

12. Add 1 mL MACS buffer to the column and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
13. Centrifuge the separated cells at 200 × *g* for 5 min and resuspend the cell pellets in 1× PBS.
14. Confirm the isolated cells by immunocytochemistry using anti-SSEA-1 antibody (Fig. 3b).

3.1.4 Immunocytochemistry of purified PGC

1. Centrifuge PGCs isolated by SDI or MACS system at 200 × *g* for 5 min and resuspend approximately 1 × 10^4 PGCs in 100 μL of 1× PBS on glass slides and dry slowly in a 37 °C slide warmer.
2. Fix the cells using 500 μL of 4% paraformaldehyde in 1× PBS at RT for 10 min. Wash the slides in 1× PBS three times.
3. Incubate with blocking solution for 10 min at RT.
4. Add anti-SSEA-1 antibody or anti-cDAZL antibody at 1:200 titers in 1 mL blocking solution and incubate the cells for 1 h at

RT under humidity using an airtight container. After 1 h, wash the slides in 1× PBS three times.

5. Add secondary antibody (e.g., goat anti-mouse IgM-PE for anti-SSEA1 antibody and mouse and anti-rabbit IgG-FITC for anti-cDAZL antibody at 1:500 dilution in 1 mL blocking solution) and incubate for 1 h at RT. After 1 h, wash the slides in 1× PBS three times.
6. Mount the slides using ProLong Gold antifade reagent (with DAPI) and analyze under a fluorescence microscope (Figs. 2b and 3b).

3.2 PGC Transplantation

1. Prepare approximately 1×10^4 isolated PGCs in 1× PBS and centrifuge at 200 × *g* for 5 min.

3.2.1 PGC Microinjection into Recipient Embryos

2. Mix the cell pellet with 2 μL PKH26 and 500 μL diluent buffer and incubate for 5 min at RT without light exposure.
3. After 5-min incubation, stop the staining by adding 1 mL of 10% FBS in DMEM and centrifuge the stained cells at 200 × *g* for 5 min.
4. Resuspend the cell pellet with 20 μL HBSS to prepare a cell density of approximately 500 cells in 1 μL medium.
5. Incubate recipient eggs with the pointed ends down until they reach HH stages 13–16.
6. Make a small window (approximately <1 cm diameter) in the pointed end of each recipient egg.
7. Remove 1–2 μL whole-blood cells from recipient blood vessel using mouth pipette (*see* **Note 4**).
8. Inject approximately 1 μL of PKH26-labeled 500 PGCs into the upper portion of the dorsal aorta of the recipient embryo.
9. Seal the window twice with parafilm, and continue incubating the eggs.

3.2.2 Migration Assay of PGCs

1. Incubate recipient eggs until HH stages 28–30 at 37 °C in air with 60–70% relative humidity.
2. Dissect embryonic gonads from recipient embryos at embryonic day 6.
3. Retrieve gonads from the recipient embryos and count the number of fluorescent PGCs in each gonad under a fluorescence microscope (Figs. 2c and 3c).

3.3 Identification and Testcross of Germline Chimera

1. Collect semen from sexual maturated WL recipients (putative germline chimera; if the donor KO PGCs were transplanted to WL recipient).
2. Isolate genomic DNA from collected semen using a DNA Purification Kit.

3. Perform breed-specific PCR using allele-specific primers under the following thermocycling conditions: 10 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 69 °C, 30 s at 72 °C, and finally 10 min at 72 °C (Fig. 3d).
4. For artificial insemination, introduce 50 μL semen from germline chimeric WL roosters to egg-laying KO hens.
5. Collect eggs from recipient hens at 1 day after artificial insemination and incubate the eggs with the pointed ends down until hatching at 37 °C in air with 60–70% relative humidity.
6. Donor-derived progenies can be distinguished by feather color (Fig. 3e) (*see* **Note 5**).

4 Notes

1. Incubate fertilized chicken, Japanese quail, mallard duck, and Muscovy duck eggs to HH stages 13–16 [17]. Incubate the chicken eggs for 55 h and the quail eggs for about 48 h. The duck eggs should then be incubated until their morphology is similar to that of the chicken and quail at the various stages (about 70 h for mallards and 92 h for Muscovy ducks).
2. Raise polyclonal antibodies against N-terminal peptides of cDAZL (aa 2–17, SANAEAQCGSISEDNTH) in a rabbit, followed by purification of antisera [6].
3. Add PBS to transwells and gently pipette the cells at an angle of 45° along the wall to keep the microporous membrane from the transwell and to isolate the PGCs with minimal damage.
4. Since the recipient embryos (HH 13–16) contain about 200 endogenous PGCs in whole blood [19], removing 1–2 μL whole-blood cells would be helpful for efficient germline chimera production.
5. Because of differences in their pigmentation (e.g., WL have a dominant pigmentation inhibitor gene (*I/I*) whereas KO have a recessive pigmentation inhibitor gene (*i/i*)), the progenies of germline chimera are distinguishable. Donor-derived progenies exhibit all black feathers (*i/i*), whereas hybrid progenies (*I/i*) exhibit slightly yellow feathers with small black spots.

Acknowledgment

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIP) (No. 2015R1A3A2033826).

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Chapter 20

Production of Germ-Line Chimeras in Zebrafish

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Abstract

The induction of germ-line chimerism in fish is a strategy for the reproduction of endangered or genetically valuable fish species. Chimeras can be created by transplanting a single primordial germ cell or multiple blastomeres from a donor into a sterile host embryo. When the host reaches sexual maturity, it will produce donor-originating gametes throughout its reproductive life span. This technique provides unique experimental conditions for basic biology research in model fish species like zebrafish. The success of cell transplantation relies on the effective sterilization of host embryos, the correct identification of developing germ cells, and the synchronization of migratory cues between the host and the transplanted cells. Developments in non-transgenic methods of germ cell ablation and identification have made germ cell transplantation more applicable to use in conservation and aquaculture. In this chapter, we provide a protocol for germ cell labeling by injection of chimeric RNA or FITC-dextran, the sterilization of host embryos using an antisense morpholino oligonucleotide, and two methods for producing germ-line chimeras in zebrafish: single primordial germ cell transplant and blastomere transplant.

Key words Zebrafish, Primordial germ cells, Germ cell transplantation, Blastomere transplant, Host embryo sterilization

1 Introduction

The transplantation of germ-line stem cells such as primordial germ cells (PGC), spermatogonia, oogonia, or blastula cells is a unique method for the propagation of endangered or genetically valuable fish species [1–3]. Isolating cells from an animal of interest and transplanting these cells into a host to produce a germ-line chimera have several benefits for the breeding of endangered, farmed, or experimental fish species. First, it is possible to select a host that is easier to maintain and breed in captivity than the donor animal, which can be useful when working with endangered species. Second, cells from one animal can be transplanted into multiple hosts, leading to the amplification of the donor genotype in the resulting offspring, which could be an efficient method to increase the fitness of fish intended for consumption. Third, germ cell transplantation also has applications in experimental biology using model fish

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_20,

species, such as gene banking and restoration of important strains [4]. This technique can also provide unique experimental conditions to study development in model species, such as changing the number of PGCs in chimeras to study the function of germ cells on sex differentiation in zebrafish [5]. The creation of germ-line chimeras involves three main concepts: (1) the identification of germ cells in the donor, (2) the ablation of germ cells in the host embryo, and (3) the timing of the transplantation of donor cells into the sterile host embryo.

In some teleost fish such as zebrafish, the first primordial germ cells arise through a process called preformation, in which early germ cells are specified by maternally derived mRNA transcripts found in the germplasm [6]. Among these transcripts are *vasa*, *nanos3* (*nos3*), and *bucky ball*, all of which have been used as a tool to identify germ cell formation and migration. The construction of chimeric mRNA transcripts that combine the 3′ untranslated region (UTR) of genes specific to the early germ line with a fluorescent gene leads to a fluorescent signal specific to the developing germ line. These transcripts are highly conserved; for example, chimeric RNA containing GFP and the *nos3* 3′UTR from zebrafish successfully identified PGCs in sturgeon (*Acipenser ruthenus*) [7]. A comparison between GFP-*nos3* 3′UTR mRNA and GFP-*olvas* (*Oryzias latipes vasa* homolog) 3′UTR mRNA found that *nos3* had a better ability to identify PGCs across seven different species: medaka (*Oryzias latipes*), zebrafish, pearl danio (*Danio albolineatus*), goldfish (*Carassius auratus*), loach (*Misgurnus anguillicaudatus*), herring (*Clupea pallasii*), and ice goby (*Leucopsarion petersil*) [8]. While GFP-*nos3* 3′UTR can identify PGCs in more species, it is more readily degraded, and the fluorescent signal is lost within 2 weeks. Nevertheless, the ability to use transcripts from unrelated species presents an exciting opportunity to study germ cell migration and isolate PGCs from less studied fish species.

Adequate preparation of host embryos is important for transplantation success, as remnant host germ cells may outcompete transplanted cells and result in low success rates. There are several methods to prevent germ cell development in host embryos, including the induction of triploidy [9], exposure to ultraviolet (UV) radiation [10], and knockdown of genes critical to germ cell development. The method required depends on the germ cell development in the selected species. For example, in sturgeon, early-stage PGCs arise from the vegetal pole, which, after chorion removal, naturally orientates downward, meaning that UV radiation emitted below the embryos eliminates the germ line without affecting somatic cell development [10]. However, this would not be the case in zebrafish, where PGCs arise in the animal pole. Currently, the most popular method of germ cell elimination is the injection of morpholino antisense oligonucleotides (MO). *Dead end* (*dnd*) is known to play a role in germ cell survival during

migration. Injection of *dnd*-MO has successfully ablated the germ line in zebrafish [11], loach [12], goldfish [13], and medaka [14] without inhibiting gonadal development. In zebrafish, knockdown of *dead end* results in an all-male population. The reason for this is unknown, but it indicates a role for PGCs in female gonadal development. In species where *dnd* knockdown results in all-male populations, the sex ratio can be altered by the addition of estrogen to the tank water for 1 month after 20 days of fertilization [15].

Once germ cells have been identified in a donor through the injection of a GFP-*nos3* 3′UTR mRNA, fluorescing cells are removed from donor embryos and transplanted into a sterile host under a fluorescent stereomicroscope. The success of single-PGC transplantation (SPT) is often attributed to the timing of embryo development between the donor and host. In zebrafish, PGCs are specified at four locations in the blastoderm but must migrate to the same location: the developing gonad [16]. The timing of PGC migration is species specific and must be carefully considered for successful transplantation. Generally, PGC migration is guided by the CXCR4b/SDF1 attractant pathway [17]. Therefore, for transplanted cells to successfully migrate to the gonadal ridge in the host, transplanted PGCs must still be receptive to SDF1, and host embryos must still be expressing SDF1 [18]. For this reason, it is recommended that, in zebrafish, PGCs are isolated from embryos no later than the ten-somite stage just as the cells reach the gonadal ridge, and that they are transplanted into blastula-stage embryos, which will just be initiating the germ cell migratory pathway [19]. This ensures adequate migration time for transplanted cells in the host embryo, which improves cell survival and overall transplant success.

The single-PGC transplant procedure requires a fluorescent microscope to identify PGCs during transplantation, a setup that often inhibits its use in standard laboratories. For this reason, an alternative method of PGC transplantation has been investigated. Blastomere transplantation (BT) is a type of cell transplant that can produce germ-line chimeras [19, 20]. In BT, donor embryos are injected with fluorescein isothiocyanate (FITC)-dextran, which produces embryo-wide fluorescence. At the blastula stage, cells are aspirated from the marginal region of the blastoderm where the PGCs are presumed to be located. As all donor cells are fluorescent, there is no need to use a fluorescent microscope to identify specific cells. The aspirated cells are transplanted into sterile blastula-stage host embryos at the marginal region. If PGCs were successfully aspirated from the donor embryo and transplanted into the host embryos, green fluorescence will be seen at the gonadal region of the host embryos at the prim-5 stage (24 h postfertilization at 28.5 °C). This method, while less specific, does allow for the relatively simple production of chimeric embryos if the location of PGCs is known and easily accessible at the blastula stage.

In this protocol, we describe the preparation of host and donor zebrafish embryos for both SPT and BT.

2 Materials

2.1 Preparation of the Microinjection Needle

1. Precision glass capillary tube (capacity 50 μL, length 100 mm).
2. Dual-stage glass micropipette puller.

2.2 Chorion Removal

1. Incubators set to 23 °C and 26 °C.
2. Solution 1 (pH 8.5, for dechorionation): 128 mM NaCl, 2.8 mM KCl, 1.8 mm $CaCl_2$, buffered with 10 mM TAPS and adjusted to pH 8.5.
3. Trypsin powder.
4. Liquefied albumen (from egg white) after mixing with a whipper.
5. Glass or plastic dish coated with ~2 mm layer of 1% agar.

2.3 Sterilization of the Host Embryos

1. Stereomicroscope with fluorescent viewing capabilities.
2. Manual microinjector.
3. Three-dimensional coarse manual manipulator with magnetic stand and an iron plate.
4. Blunt glass probes (*see* **Note 1**).
5. Glass pipettes with rubber bulb.
6. Extra-long tips for filling of microcapillaries.
7. Solution 1 (pH 7.5, for embryo culture): 128 mM NaCl, 2.8 mM KCl, 1.8 mm $CaCl_2$, buffered with 10 mM HEPES and adjusted to pH 7.5.
8. Glass or plastic dish coated with ~2 mm layer of 1% agar.
9. *dnd* MO (100 μM: gCTgggCATCCATgTCTCCgACCAT) in 0.2% KCl (*see* **Note 2**).
10. Liquefied albumen (from egg white) after mixing with a whipper (*see* **Note 3**).
11. Penicillin (final conc. 0.01%).
12. Streptomycin (final conc. 0.01%).
13. Incubator set at the optimal temperature for embryo development.

2.4 Preparation of Needles for Cell Transplantation

1. Precision glass capillary tube (capacity 50 μL, length 100 mm).
2. Dual-stage glass micropipette puller.
3. Micropipette grinder.

2.5 Transplantation of Single Primordial Germ Cell (SPT) and Blastula Cells (BT)

1. Stereomicroscope with fluorescent viewing capabilities.
2. Manual microinjector.
3. Three-dimensional coarse manual manipulator with magnetic stand and an iron plate.
4. Glass needles for cell transplantation.
5. Blunt glass probes.
6. Glass pipettes with rubber bulb.
7. Solution 1 (pH 7.5, for embryo culture): 128 mM NaCl, 2.8 mM KCl, 1.8 mm $CaCl_2$, buffered with 10 mM HEPES and adjusted to pH 7.5.
8. Dissociation solution (0.1% trypsin, 0.5% trisodium citric acid dihydrate, 0.05% DNase in Solution 1, pH 7.5).
9. Glass or plastic dish coated with ~2 mm layer of 1% agar.
10. 5% FITC-dextran (M.W. 10,000) in 0.2% KCl.
11. GFP-*nos3* 3′UTR mRNA (300 ng/μL in 0.2% KCl) (*see* **Note 4**).
12. Liquefied albumen (from egg white) after mixing with a whipper (*see* **Note 3**).
13. Penicillin (final conc. 0.01%).
14. Streptomycin (final conc. 0.01%).
15. Solution 2: 1.8 mm $MgCl_2$, 1.8 mm $CaCl_2$.
16. Incubator set at the optimal temperature for embryo development.

3 Method

3.1 Preparation of the Microinjection Needle

1. A 50uL micropipette is pulled to make two needles using a dual-stage glass micropipette puller to produce a slender, tapered needlepoint as shown in Fig. 1a.

3.2 Chorion Removal

1. Collect fertilized zebrafish eggs from a spawning tank.
2. Wash the one-cell-stage zebrafish embryos in Solution 1 (pH 8.5).
3. Remove the wash solution, and replace it with Solution 1 (pH 8.5) with 0.1% trypsin to begin chorion removal.
4. Chorion removal will take approximately 10–30 min (*see* **Note 5**). The chorions will begin to lift, appearing as a second ring around the embryo. Gently agitate the dish to remove the chorions completely.

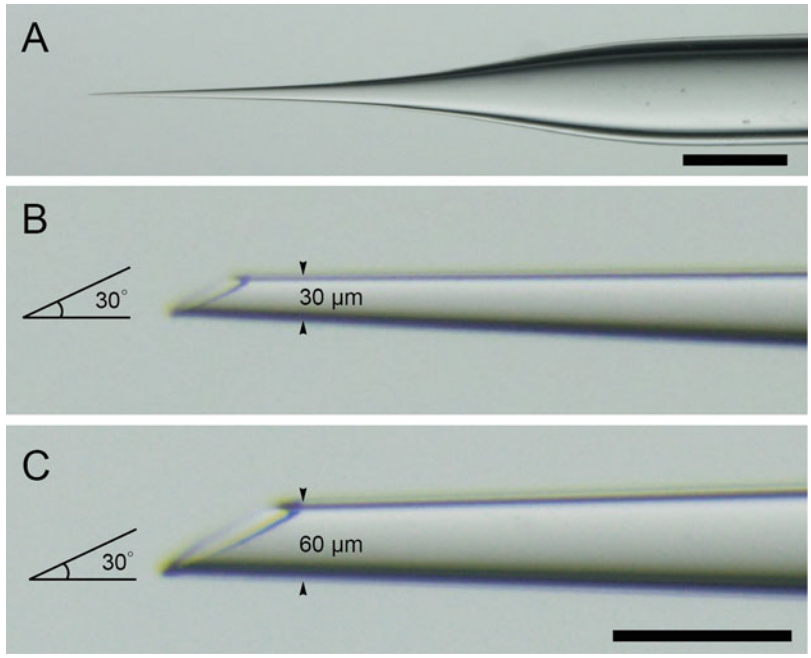

Fig. 1 The shape of needles for microinjection (**a**), SPT (**b**), and BT (**c**). The scale bars in (**a**) and (**c**) indicate 1 mm and 100 μm, respectively

5. Transfer the dechorionated embryos to a 1% agar-coated plate containing Solution 1 (pH 7.5) supplemented with 1.6% albumen (*see* **Note 6**).

3.3 Sterilization of the Host Embryos

1. Arrange the dechorionated one-cell-stage embryos in a single line on an agar-coated plate containing Solution 1 (pH 7.5) supplemented with 1.6% albumen (Fig. 2a).
2. Load a pulled 50uL micropipette with the *dnd*-MO solution.
3. Connect the injection needle to the microinjector, and place an agar-coated dish filled with Solution 1 (pH 7.5) on the stage of a stereomicroscope.
4. Move the tip of the injection needle into the Ringer's solution carefully. Gently break the tip of the injection needle by grazing the tip with the microloader tip used to load the micropipette (Fig. 2b).
5. Inject the *dnd*-MO solution into the yolk cell of each embryo (Fig. 2c) (*see* **Note 7**).

3.4 Preparation of Needles for the Cell Transplantation

1. A 50uL micropipette is pulled to make two needles using a dual-stage glass micropipette puller.
2. Pulled micropipettes are beveled using a micropipette grinder.

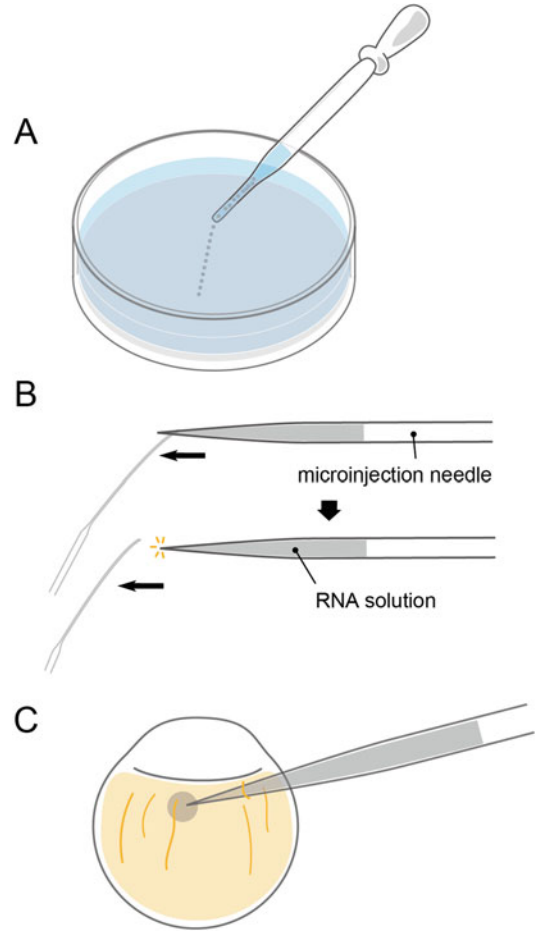

Fig. 2 Schematic illustration of a microinjection into zebrafish eggs. (**a**) Align the one- to four-cell-stage embryos in a single line using a glass pipette on the agar-coated dish filled with Solution 1. (**b**) Place the tip of the microinjection needle into Solution 1 in the dish, and break the needle tip by grazing with the microloader tip under the stereomicroscope. (**c**) Microinject the RNA solution into the yolk just under the blastodisc

3. Bevel sizes vary depending on the cell type and species to be transplanted. For zebrafish PGC transplantation, a bevel size of 30 μm is recommended, and for blastula cell transplantation, a bevel size of 60 μm is recommended (Fig. 1b, c).

3.5 Transplantation of a Single Primordial Germ Cell (SPT)

3.5.1 Visualization of Primordial Germ Cells

1. Arrange the dechorionated one- to four-cell-stage embryos in a single line on an agar-coated dish containing Solution 1 (pH 7.5) supplemented with 1.6% albumen.
2. Load a pulled 50 μL pipette with GFP-*nos3* 3′UTR in 0.2 M KCl.
3. Connect the injection needle to the microinjector, move the tip of the injection needle into the Ringer's solution, and break the tip as described in Subheading 3.3.
4. Transfer the injected embryos into individual wells on a 96-well plate containing 7.5 pH Ringer's solution supplemented with 1.6% albumin, 0.01% penicillin, and 0.01% streptomycin.
5. Keep the embryos in a 23 °C incubator overnight (*see* **Note 8**).
6. The next day, before the transplantation, transfer the developing embryos (one- to five-somite stages) to an agar-coated dish filled with Solution 1.
7. Check the embryos for fluorescent cells under a fluorescent microscope, and select embryos with well-labeled PGCs and low GFP background expression.

3.5.2 Isolation of Primordial Germ Cells

1. Place the GFP-*nos3* 3′UTR-injected 10- to 15-somite-stage embryos into the 1.5 mL microcentrifuge tube.
2. Remove any solution that has been transferred with the embryos.
3. Add the cell dissociation solution 10 μL per one embryo in the microcentrifuge tube (*see* **Note 9**).
4. Pipette the embryos gently to dissociate the cells with a 20–200 μL pipette (*see* **Note 10**), and then keep the cell solution on ice until the transplantation (*see* **Note 11**).
5. Set a dish containing Solution 1 (pH 7.5) supplemented with 1.6% albumen on the stage of a fluorescent stereomicroscope.
6. Take a portion (5–10 μL) of the cell solution using a 2–20 μL pipette, and expel gently onto an uncoated dish containing Solution 1 (pH 7.5) with 1.6% albumen.
7. View the dish under a fluorescent stereomicroscope. Primordial germ cells can be easily identified by green fluorescence.

3.5.3 Transplantation of a Single Primordial Germ Cell into a Host Embryo

1. Fill an uncoated dish with Ringer's solution 1 (pH 7.5) supplemented with 1.6% albumen.
2. Transfer the dechorionated blastula-stage host embryos and dissociated cells prepared from GFP-*nos3* 3′UTR-injected donor embryos (*see* **Note 12**).
3. Attach a beveled 30 μm diameter microneedle to the microinjector, and push the oil line up to where the needle begins to taper off.

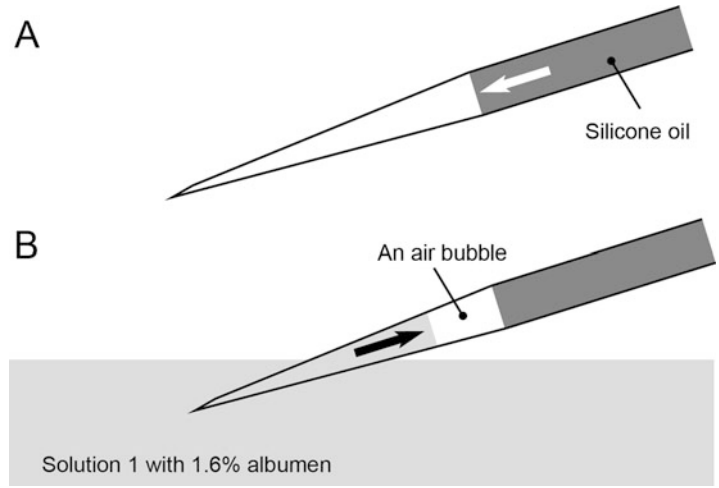

Fig. 3 How to stabilize the inner pressure of the transplantation needle. (**a**) Push the silicone oil line up to where the needle begins to taper off. (**b**) Place the needle tip into a dish filled with Solution 1 for at least 2 min, and let the solution fill the tip of the needle by capillary action

4. Place the tip of the needle into the Ringer's solution with the bevel facing toward the top of the dish, and leave for at least 2 min to stabilize the inner pressure of the needle until the solution fills the needle tip by capillary action (Fig. 3a, b).
5. Move the needle tip carefully to the single fluorescing cell, and slowly aspirate the cell into the needle (Fig. 4a).
6. Hold the single cell toward the tip of the needle, and position a blastula-stage host embryo next to the needle (Fig. 4b).
7. Use a blunt glass probe to hold the host embryo in place while piercing the marginal region of the host embryo with the injection needle (*see* **Note 13**).
8. Slowly push the single cell into the embryo, and then withdraw the injecting needle (Fig. 4c, d) (*see* **Note 14**).
9. Transfer the transplanted embryos into separate wells of a 96-well plate containing Solution 1 (pH 7.5) with 1.6% albumen, 0.01% penicillin, and 0.01% streptomycin.
10. Incubate overnight at 26 °C.
11. Move developing embryos (prim-5 stage) to an agar-coated dish filled with a Solution 1 (*see* **Note 15**).
12. Observe embryos under a fluorescent microscope to check the location of GFP-labeled PGC (*see* **Note 16**).
13. Transfer the embryos with GFP-labeled PGCs at the gonadal region to a new 96-well plate filled with Solution 2 containing 0.01% penicillin and 0.01% streptomycin until the feeding stage.

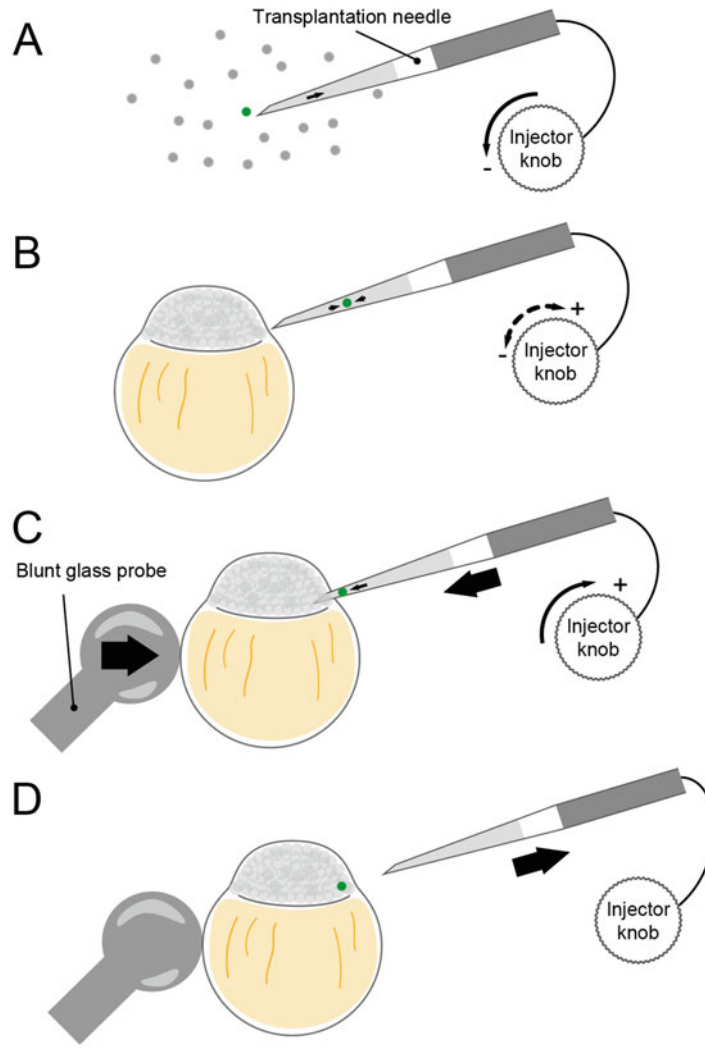

Fig. 4 Schematic illustration of SPT. (**a**) Find a PGC under a fluorescent stereomicroscope, and aspirate into the needle by applying negative pressure with the injector. (**b**) Hold the PGC in the middle of the needle by making fine adjustments of the injector pressure, and bring the needle near a host embryo. Place the needle tip just on the marginal region of the blastodisc (without punching). (**c**) Slowly move the PGC toward the needle tip by applying a slight positive pressure with the injector. As the PGC approaches the needle tip, insert the needle into the marginal region of the host blastodisc by pushing both the embryo and the needle. (**d**) Place the PGC at the marginal region and pull the needle out from the embryo

3.6 Transplantation of Blastula Cells (BT)

3.6.1 Fluorescent Labeling of Donor Embryos

1. Arrange dechorionated one-cell-stage embryos in a single line on an agar-coated plate containing Solution 1 (pH 7.5) supplemented with 1.6% albumen.
2. Load a pulled microneedle with 5 mM fluorescein isothiocyanate (FITC)-dextran (molecular weight of 10,000) in 0.2 M KCl.
3. Attach the microneedle to the microinjector. Break the needle tip as described in Subheading 3.3.
4. Inject into the yolk cell just under the blastodisc of each embryo.
5. Confirm fluorescence by viewing on a fluorescent stereomicroscope.

3.6.2 Transplantation of Blastula Cells (BT)

1. Place blastula-stage donor and host embryos on separate sides of the same agar-coated dish containing Ringer's Solution 1 (pH 7.5).
2. Attach a beveled 60 μm diameter microneedle to the microinjector, and push the oil line up to where the needle begins to taper off.
3. Place the tip of the needle into the Ringer's solution with the bevel toward the top of the dish, and leave to stabilize for at least 2 min, as described in Subheading 3.5.3.
4. Position the needle with the bevel pressed against the marginal region of the blastula of a FITC-injected donor embryo, where PGCs are expected to be localized.
5. Aspirate roughly 10–50 cells (depending on stage) from the marginal region of the donor embryo (Fig. 5a) (*see* **Note 17**).
6. Hold the blastula cells in the middle of the needle, and move the needle next to a host embryo. Hold the host embryo in place with a blunt glass probe, and transfer the donor cells into the marginal region of the host blastula embryo (Fig. 5b, c).
7. Reposition the donor embryo to aspirate cells on the opposing side of the blastula. Repeat **steps 4** and **5** with a new host embryo to increase the chance of transplanting PGCs (Fig. 5d) (*see* **Note 18**).
8. Transfer the transplanted embryos into separate wells of a 96-well plate containing Ringer's Solution 1 (pH 7.5) supplemented with 1.6% albumen, 0.01% penicillin, and 0.01% streptomycin.
9. Incubate overnight at 26 °C.
10. Observe and check the location of FITC-labeled PGCs in the embryos as described in Subheading 3.5.3.

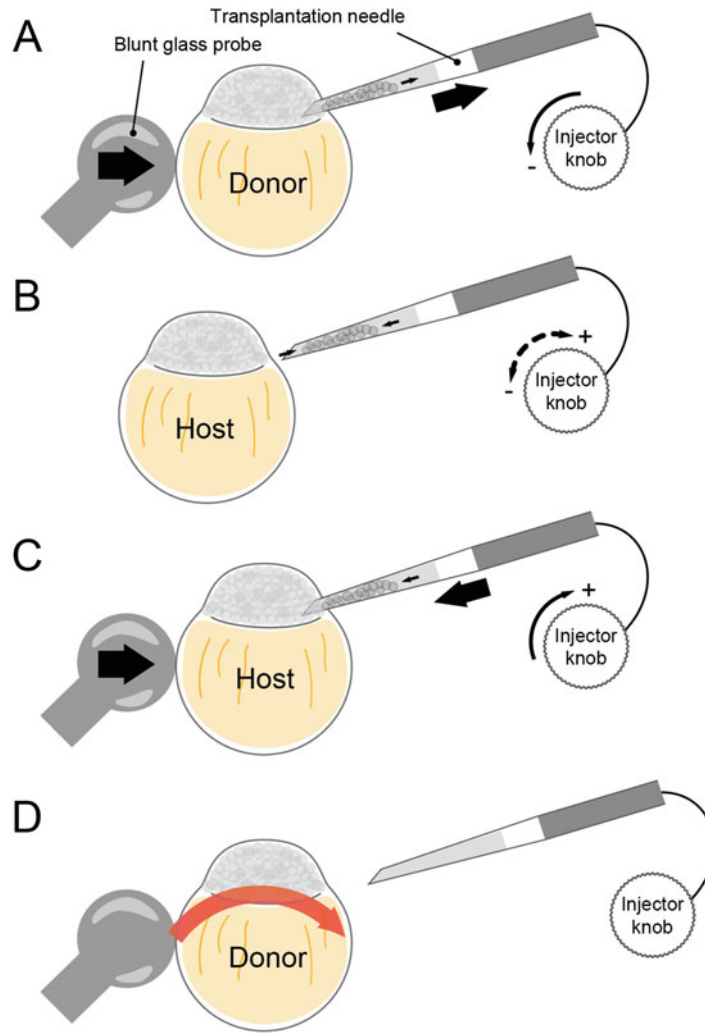

Fig. 5 Schematic illustration of BT. (**a**) Aspirate the blastomeres from the donor embryo under a stereomicroscope by applying negative pressure with the injector. (**b**) Hold the cells in the middle of the needle by making fine adjustments of the injector pressure, and bring the needle near a host embryo. Place the needle tip just on the marginal region of the blastodisc (without punching). (**c**) Slowly move the cells toward the needle tip by applying a slight positive pressure with the injector. As the cells approach the needle tip, insert the needle into the marginal region of the host blastodisc by pushing both the embryo and the needle. Push the cells into the marginal region, and then remove the needle from the embryo. (**d**) Flip the donor embryo to aspirate cells from a different side of the blastodisc, and then repeat the transplantation

11. Transfer the embryos to a new 96-well plate filled with Solution 2 containing 0.01% penicillin and 0.01% streptomycin until the feeding stage.

4 Notes

1. We use a 1 mL disposable glass Pasteur pipette to make the blunt glass probe with a small gas burner.
2. The sequence of the morpholino antisense oligonucleotide against *dead end* mRNA was obtained from Ciruna et al. [20].
3. Whip the egg white just until it foams loose peaks, and wait for 10–15 min to get liquefied albumen on the bottom.
4. GFP-*nos3* 3′UTR was originally designed by Köprunner et al. [21].
5. The time for chorion removal depends on the quality of eggs, stage of embryos, and titer of the enzyme.
6. The supplementation of 1.6% albumen is meant to inhibit trypsin activity.
7. The microinjection needs to be done by the eight-cell stage for complete sterilization.
8. A lower temperature (23 °C) decreases the developmental rate and allows for the proper stage of development at a convenient time.
9. We usually collect ten embryos and add 100 μL dissociation solution in a tube.
10. If few embryos are used as donors, use a 2–10 μL pipette for dissociation. Gently mix the cell suspension to avoid damage of the cells by drawing air bubbles into the pipette. Treated cells can be checked by microscopy to make sure that they are dissociated correctly.
11. The cell solution can be kept on ice for up to 2 h.
12. For PGC transplantation, 1 k-cell to sphere-stage (3–4 h post-fertilization at 28.5 °C) embryos are used as the host.
13. In normal embryonic development, PGCs are specified at the marginal region of the blastodisc. In our experience, the success rate of donor PGC migration to the genital ridge is higher when the PGC is transplanted in this region.
14. Keep the needle inside the embryo for a few seconds after extruding the cell to make sure that the cell remains in the blastodisc.
15. Embryos must be observed before the pigmentation stage. Pigment cells can veil the transplanted PGCs at the genital ridge region, making visualization of PGCs difficult.
16. In our laboratory, generally 30–40% of transplanted embryos possess donor PGCs at the gonadal region.

17. In this technique, somatic cells are also labeled with FITC, and PGC transfer is simply confirmed by its location at the genital ridge at the prim-5 stage. Thus, if too many cells are transplanted, it will be difficult to confirm FITC-labeled PGCs due to high background FITC fluorescence from the somatic cells.
18. In our experience, donor embryos from which blastomeres have been aspirated can develop normally with high survivability.

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Chapter 21

A Method for Zebrafish Follicle Transplantation into Recipient Mothers for the Generation of Fertilizable Eggs and Viable Offspring

Zsolt Csenki and Ferenc Mueller

Abstract

In zebrafish and other externally developing animals, maternal effect gene products significantly contribute to embryonic and larval development. Several methods exist for both forward and reverse genetic manipulation of maternal gene products, yet it remains technically difficult to interfere with maternal gene products. Therefore, alternative methods to manipulate maternal factors in oocytes remain of interest. Here we describe a method which allows manipulation and subsequent transplantation of stage I–II follicles of zebrafish into recipient mothers where donor oocytes can develop into mature eggs and produce viable offspring. Additionally, we describe a simple microinjection protocol for injecting reporter constructs into follicles as a proxy for manipulating maternal effect genes.

Key words Oogenesis, Follicle, Transplantation, Transgenic, Maternal effect

1 Introduction

In zebrafish and other anamniotes, maternal gene products (RNA and proteins deposited in the egg) drive early embryogenesis almost exclusively until the mid-blastula transition (MBT), when the zygotic genome switches on and start contributing to the regulation of developmental programs [1, 2]. Maternal contribution to embryogenesis continues to be essential for development (reviewed in [3]). The extent to which maternal effects contribute to embryonic and larval development is hard to estimate due to the scarcity of functional data on maternal gene products. Despite the fact that thousands of genes are expressed maternally [4] and an unknown number of different proteins are also inherited from the mother the genetic and molecular mechanisms of how they affect development and how maternal factors give way to zygotic RNAs remain little understood. This is due to the difficulty of interfering

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_21,

with maternal gene products either by forward or reverse genetic techniques.

A technology which allows interference with maternal gene function is necessary to address the requirement of maternal gene products to oogenesis and embryogenesis. Fourth-generation maternal effect mutant screens [5, 6] and gynogenesis-based screen for recessive genes by diploidization of haploid mutation-containing genomes [5] are available. The generation of maternal mutants from zygotic mutant lines can be achieved by phenotypic rescue using wild-type mRNA injection into zygotic mutants which may result in viable, egg-producing homozygous recessive females [7], or by germ-line replacement [8]. Both these approaches depend on the availability of a zygotic mutant, which can now be generated by cas9/CRISPR-targeted mutagenesis [9]. A promising reverse genetic alternative will be the use of conditionally expressed knockdown and knockout technologies utilizing oocyte-specific activation of genome-manipulating recombinases or nucleases.

Given the very limited information on the genetic mechanisms involved in oogenesis and on maternal contribution to embryo development, and the difficulty to manipulate the genes involved in these processes with conventional technologies, the development of new approaches to manipulate oocytes remains of interest. While it is possible to isolate and in vitro maturate stage IV oocytes in zebrafish, early stage I–II oocytes have not yet been shown to be cultured and induced to grow efficiently.

We describe here a proof-of-principle method, which has been first reported in [10] and which offers an alternative approach to manipulate early-stage zebrafish oocytes with the long-term goal of interference with maternal effect gene products during oogenesis. We describe how stage I–II oocytes of zebrafish can be recovered and transplanted into recipient mothers where these stage I–II oocytes integrate into the ovary and can develop normally to produce fertilizable eggs. These stage I–II oocytes can be microinjected with RNA and DNA solutions, which allows detection of reporter gene expression paving the way to further developing tools for interfering with maternal gene activities through the micromanipulation of oocytes.

2 Materials

2.1 Collection and Transplantation of Donor Follicles

1. *Tricaine solution*: Tricaine powder (ethyl 3-aminobenzoate methanesulfonate, CAS Number: 886-86-2) dissolved in distilled water (pH 7, adjusted by using 1 M Tris, pH 9) to generate a stock solution of 3% was used to dilute to a final concentration of 0.003% with clean fish-holding system water.

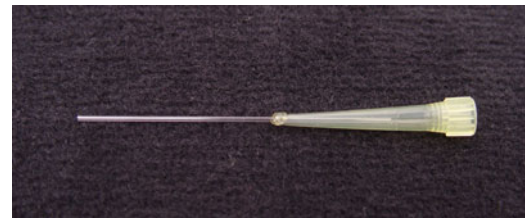

Fig. 1 Modified pipette tip for transplantation

2. *Zebrafish Ringer*: 116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES (pH 7).
3. *mPBS*: 2.7 mM KCl, 137 mM NaCl, 1 mM NaH_2PO_4, 3.2 mM $Na_2HPO_4 \times 2H_2O$.
4. *70% Ethanol.*
5. *Petri dish for suspending the ovary*: 1.5–2% molten agarose in zebrafish Ringer solution, poured into a Petri dish and let to congeal.
6. *Follicle-collecting Petri dish*: Pour 1.5–2% molten agarose prepared in zebrafish Ringer solution into a Petri dish. Carefully lay injection plate mold (e.g., Midsci FM-600) on top. Avoid the formation of air bubbles. After the agarose is congealed, remove the mold.
7. *Modified pipette tip for transplantation*: Take a blunt glass capillary without modification (length 7–8 cm, external diameter 1.2 mm, internal diameter 0.65 mm (e.g., Narishige G-1 or G-1.2) and a plastic pipette tip (200 μL)). Cut the tip of the pipette tip so that the capillary fits inside it. Insert the capillary into the trimmed pipette tip and melt gently the two pieces together over flame (e.g., gas lighter) to make a tight seal so that the capillary can be used through the action of the automatic pipette (Fig. 1) (*see* **Note 1**).
8. *Fish-positioning sponge*: Place a 2–3 cm thick sponge disk into the bottom of a 10 cm diameter glass Petri dish (same size in diameter). Cut a 6–7 cm long 1–1.5 cm deep groove into the center of the sponge by using a scalpel. Moisten the sponge with clean aquarium water before putting fish into the trench.

2.2 Microinjection of Oocytes

1. *Petri dish for injection of oocytes*: Pour 1.5–2% molten agarose dissolved in zebrafish Ringer solution into a Petri dish, and let it congeal. Cut a 2 mm deep groove into the agar using a scalpel. One wall of the groove should be vertical and the other should be at an angle of 25–30° to the vertical wall.

3 Methods

3.1 Preparing the Recipient Females

1. Breed the recipient females on the day of transplantation by using the usual natural breeding method of the laboratory (e.g., pair matings set up the day before the day of transplantation). Only females which laid at least 50 eggs can be used as recipients in the experiment to secure emptying the ovary before transplantation (*see* **Note 2**).

3.2 Isolation and Collection of the Donor Follicles

Collection of follicles can be carried out in parallel with breeding.

1. Anesthetize the donor females terminally (e.g., overdose tricaine until cessation of gill movement for 15 min or as in your animal protocol), then remove the ovaries surgically, and place them in mPBS solution for 3–5 min (*see* **Note 3**).
2. Remove the ovaries from the mPBS solution and cut into small pieces (e.g., blocks of approximately 5 mm diameter).
3. Place the ovary pieces on an agarose-coated Petri dish containing zebrafish Ringer solution and suspend using a Pasteur pipette. During suspension, the follicles separate from the connective tissue of the ovary.
4. Collect the desired size follicles using a Pasteur pipette and transport them to the follicle-collecting Petri dish filled with zebrafish Ringer solution. Place the follicles next to each other in the agar groove as close as possible. For both transplantation and other manipulations stage I and stage II follicles seem to be the most suitable (*see* **Notes 4** and **5**).
5. If there are not enough follicles of the expected size (stage I–II follicles, size range between 7 and 340 μm) in the Petri dish, resuspend or use another donor female.

3.3 Transplantation Method

1. After breeding, anesthetize the recipients with tricaine solution.
2. Place the fish on the sponge and fix the position so that the genital papilla should be easily accessible (Fig. 2) (*see* **Note 6**).
3. Disinfect the belly of the fish by using a cotton swab dipped in 70% ethanol/swabbing with ethanol.
4. Rinse the ovaries twice in zebrafish Ringer solution with the modified pipette tip also used for transplantation (similarly to point 6). Disinfect the pipette tip with 70% ethanol before rinsing (*see* **Note 7**).
5. Suck the collected follicles into the prepared pipette tip with an automated pipette from the oocyte-collecting Petri dish. Approximately 25–30 μL solution can be dispensed into the

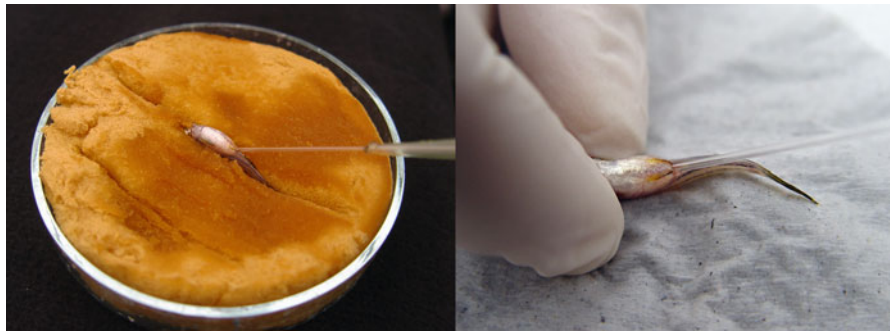

Fig. 2 Proper positioning of the recipient female in a sponge and by freehand

Fig. 3 Asterisks indicate the target area for positioning the transplantation needles during the procedure

recipient ovary at a time and around 25 to 50 follicles fit in such volume.

6. Insert the glass capillary 5–8 mm deep into the oviduct through the genital papilla of anesthetized females. Inject oocytes into the front third area of the ovary (Fig. 3) (*see* **Note 8**).
7. Repeat the procedure with the other ovary if necessary (Fig. 3).

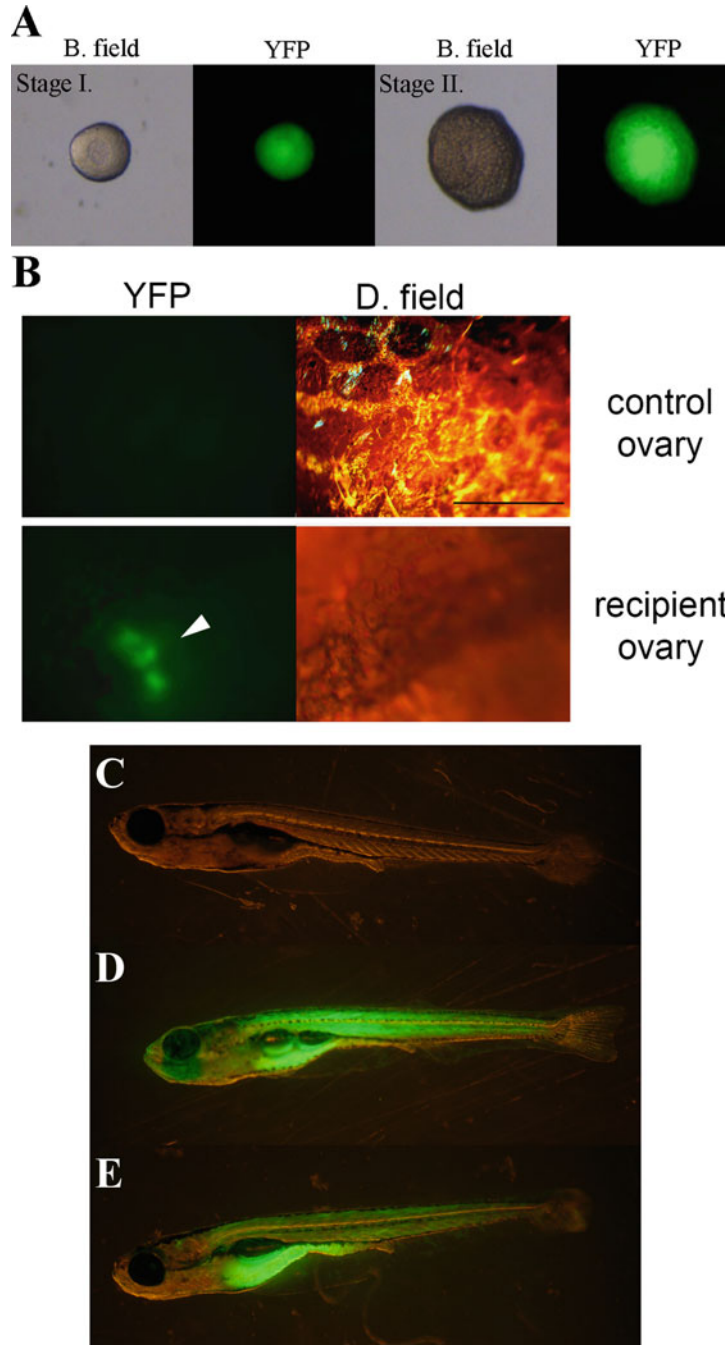

Fig. 4 Intraovarian transplantation of stage I–II follicles results in viable zebrafish embryos. (**a**) Isolated stage I and stage II donor transgenic (β-actin: yfp) follicles prior to transplantation. (**b**) In vivo observation of recipient ovaries 2 days after/on the second day following transplantation. Fluorescent signal of transgenic follicles was clearly visible through the abdomen of anesthetized recipient *gold* females (arrowhead). No fluorescent signal was detected in the ovary of control

8. Release the transplanted female into fish water to regain consciousness and keep separately for 1 day before transferring into communal tanks in normal housing conditions (*see* **Notes 9** and **10**).

3.4 Examination of Recipient Females

1. Breed the transplanted females weekly by following the usual natural breeding method of the lab. The first embryos from the transplanted follicles may appear 3 weeks after transplantation (Fig. 4) (*see* **Note 11**). Recipient females should be propagated for maximum 6–8 weeks following transplantation. The efficiency of the method is rather low. Only 2% of transplanted females are expected to have donor offspring (0.04% of the donor follicles turn into eggs).

3.5 Microinjection of Oocytes

The following protocol is optional and offers the manipulation of oocytes by microinjection of reagents such as RNA or morpholino antisense oligonucleotides, prior to reintroduction into recipient females.

1. Isolate the follicles as described above.
2. Collect follicles of the desired size in a Petri dish for injection filled with zebrafish Ringer solution.
3. Arrange the follicles side by side in a row in the groove of the Petri dish, without covering. In the groove, the follicles are placed in a row for the injection.
4. Inject the oocytes by targeting the nucleus. The sloping side of the groove should be toward your injecting hand. Microinjection can be carried out under a stereomicroscope with a pressure microinjector either by freehand or by using a micromanipulator (*see* **Note 12**).
5. Transplant the injected oocytes as described above, or incubate them in the Petri dish until further examinations (Fig. 5).

Fig. 4 (continued) gold females. (**c**) Non-transgenic offspring of the recipient female mated with a wild-type male. (**d**) Transgenic offspring of a sibling of the donor female mated with a wild-type male. (**e**) Transgenic offspring originated from a recipient female, injected with transgenic donor follicles and mated with wild-type male. All larvae are shown at the age of 10 days postfertilization. The activity of the transgene is well detectable under YFP filter in the positive control and in the muscle of the fry derived from donor follicles. The signal is undetectable in the offspring of the recipient female developed from her own follicle

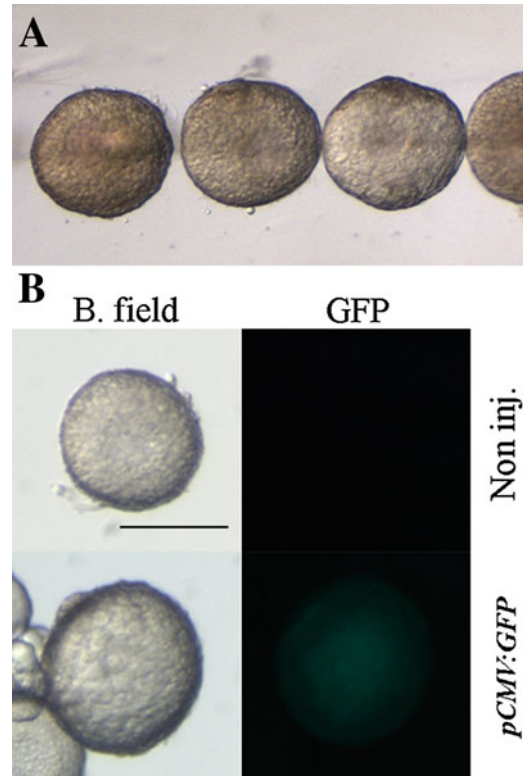

Fig. 5 Microinjected stage II follicles express reporter protein. (**a**) *pCMV:GFP* DNA-microinjected oocytes are placed side by side in a row in the groove of the Petri dish. (**b**) Injected oocytes were incubated in vitro at 27.5 °C in zebrafish Ringer solution for 24 h after microinjection. GFP activity was observed in the microinjected oocytes

4 Notes

1. Disinfect the capillary with 70% ethanol before it touches the body of the fish.
2. Alternatively, older females with larger bodies should be selected as recipients for easier targeting of ovaries in them.
3. Pretreatment with mPBS solution can be omitted if the donor's ovaries are loose. In this case, after carving the ovaries, suspending the ovaries is sufficient to detach follicles.
4. Stage I oocytes range from 7 to 140 μm in diameter, are transparent with visible germinal vesicle, and form follicles. In the following stage II (140–340 μm) accumulation of proteins required for embryogenesis takes place. In this stage the oocyte becomes more opaque and the germicidal vesicle in the middle becomes increasingly difficult to observe [11].

5. Follicles should not be kept in Ringer solution for more than 90 min at room temperature.
6. Instead of using a sponge, a wet paper towel is also sufficient to fix the position of the fish. In this case, carefully hold the fish with your fingers and position the body.
7. Rinsing of the ovaries is a risk of injury to the internal organs of the mother and can be omitted if eggs were stripped from the fish.
8. A few hours after transplantation the oocytes are well distributed within the ovary; therefore it is sufficient to transplant the oocytes only to one side of the abdomen.
9. Extra care needs to be taken when fish is waking up from anesthesia. Monitor the behavior of the fish during the procedure. If the fish does not wake up after an extended period of time or gets visibly distressed or injured during the procedure the animal needs to be euthanized by strictly following the appropriate animal welfare laws.
10. Immediately after transplantation, follicles may leak out of the ovary; therefore care needs to be taken when moving the fish.
11. Weekly breeding boosts oocyte maturation and secures regular egg production.
12. Depending on the size of the oocyte, 0.1–0.2 nL of solution may be injected per oocyte.

Acknowledgments

The publication of this chapter is supported by the EFOP-3.6.3-VEKOP-16-2017-00008 project co-financed by the European Union and the European Social Fund. The authors thank Francisco J. Pelegri for critical reading of this chapter.

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Chapter 22

Optimized Protocol of Zebrafish Somatic Cell Nuclear Transfer (SCNT)

Sukumal Prukudom, Kannika Siripattarapravat, William Poulos, and Jose B. Cibelli

Abstract

Zebrafish (*Danio rerio*) is an established animal model to study developmental biology as well as a wide array of human diseases. Here we describe a protocol for somatic cell nuclear transfer (SCNT). This protocol can be used to introduce genetic modifications in zebrafish and for the study of cell plasticity.

Key words Zebrafish, Somatic cell nuclear transfer, Cloning

1 Introduction

Zebrafish (*Danio rerio*) was cloned using somatic cells for the first time in 2002 [1]. Our laboratory published an optimized protocol for zebrafish somatic cell nuclear transfer (SCNT) in 2009 [2]. More improvements were also reported in our subsequent articles [3, 4].

Here we have written a protocol for zebrafish SCNT that covers multiple aspects of the procedure. Our intention is to have an easy-to-follow description of the steps involved. These include strains of zebrafish to use, breeding practice of the fish, cell isolation and culture of the donor cells, micromanipulation, gamete collection, in vitro fertilization (IVF), preparation of the egg's holding media, selection and enucleation of unfertilized eggs, preparation of donor cells and injection into the eggs, management of the reconstructed eggs, training schedule, and identification of clones and materials required.

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_22,

2 Materials

2.1 Selection of Zebrafish Strains to Use

1. Zebrafish strains for supplying eggs: TAB hybrid zebrafish, an F1 cross between zebrafish AB and Tubingen strains.
2. Zebrafish strains used for supplying nucleus-donor cells.

2.2 Fundamental Practices of Working with Embryos and Adult Zebrafish used for SCNT

1. Mating boxes: 10–20 sets.
2. Hanks' balanced salt solution (HBSS): 9.7 g/L Hanks' balanced salts, pH 7.0 with addition of 0.35 g of sodium bicarbonate and sterilized with a 0.22 μm filter and kept at 4 °C.
3. Embryo water: 10% v/v of Hanks' balanced salt solution.
4. Methylene blue: 10 ppm in embryo medium.
5. Bleach solution: 0.05% Sodium hypochlorite in embryo medium.

2.3 In Vitro Fertilization and Utilization of Egg-Holding Media

1. Mating boxes: 10–20 sets.
2. Kimwipe tissue paper.
3. Smooth-tipped glass rod.
4. Four Cosmetic sponge.
5. 5 μL Glass pipette.
6. Strainer.
7. Refrigerated centrifuge.
8. 0.22 μm Filter.
9. Tricaine stock solution (MS222): 0.4% w/v embryo medium, pH 7.0. Stock solution must be kept in −20 °C and replaced every 6 months.
10. Tricaine working solution: 0.02% w/v in embryo medium, pH 7.0.
11. Egg-holding media: Pure Chinook salmon ovarian fluid (CSOF), sterilized with a 0.22 μm filter.
12. H-BSA: 0.5% w/v of bovine serum albumin in HBSS, sterilized with a 0.22 μm filter and kept at 4 °C.

2.4 Essential Procedures for SCNT

2.4.1 Preparation of Micromanipulation

1. Borosilicate glass microtube: Outside diameter of 1 mm and inside diameter of 0.58 mm.
2. Injecting needle: ICSI pipette with 5–10 μm inner diameter.
3. Diamond cutter.
4. Pipette puller machine.
5. Sand papers: Fine P400 and extra fine P2000.
6. Microforge machine.

7. Polyvinylpyrrolidone in CSOF: 5% w/v of polyvinylpyrrolidone in CSOF. Keep at 4 °C overnight, and then filter with a 0.45 μm filter, followed by a 0.22 μm filter. Keep in 500 μL aliquots, at −80 °C until use.
8. Polyvinylpyrrolidone in DMEM: 1% w/v of polyvinylpyrrolidone in Dulbecco's modified Eagle medium (DMEM). Keep at 4 °C overnight and then filter with a 0.45 μm filter, followed by a 0.22 μm filter. Keep in 500 μL aliquots, at −80 °C until use.
9. Mineral oil, cell culture grade.
10. Fluorinert.
11. Fluorescent inverted microscope equipped with UV light source.
12. Pipette controller: Two sets (left- and right-hand side).
13. Micromanipulator arms: Two sets (left- and right-hand side).
14. CellTram Air microinjector.
15. CellTram vario microinjector.

2.4.2 Enucleation Practice

1. Materials for gamete collection (*see* Subheading 2.3).
2. Materials for micromanipulation (*see* Subheading 2.4.1).
3. Laser objective lens: 40×.
4. Laser-controlling system: Operating software and manual switch (Hamilton Thorne, Beverly, Massachusetts).
5. Hoechst stock solution: 50 mg/mL in HBSS.
6. Hoechst staining solution: 50 μg/mL in CSOF.
7. Materials for in vitro fertilization (*see* Subheading 2.3).

2.4.3 Preparation of Nucleus-Donor Cells

1. Rinsing solution: 1% v/v antibiotic in LHC (laboratory human carcinogenesis, e.g., Gibco) media.
2. Fine-tip forceps.
3. Surgical blade.
4. Polyvinylpyrrolidone in serum-free DMEM: 2% w/v of polyvinylpyrrolidone in serum-free DMEM media.
5. Phosphate buffer solution (PBS): 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4.
6. Trypsin-EDTA: 0.025% w/v in serum-free PBS.
7. TrypL-Express Enzyme (Thermo Fisher).
8. Fetal bovine serum in DMEM: 10% v/v in DMEM media.
9. Hypotonic solution: 0.6% w/v NaCl in PBS.

2.5 Workflow Process of SCNT and Post-Activation Management

2.5.1 Microinjection of the Somatic Cell Nucleus Through the Micropyle

1. Holder pipette: 500 μm of an inner diameter glass pipette.
2. Supporting pipette: 20 μm of an inner diameter glass pipette.
3. Injecting needle: 5–10 μm of an inner diameter ICSI pipette.
4. Polyvinylpyrrolidone in CSOF: 5% w/v of polyvinylpyrrolidone in CSOF.
5. Polyvinylpyrrolidone in DMEM: 1% w/v of polyvinylpyrrolidone in DMEM media.
6. Mineral oil, cell culture grade.
7. Fluorinert.
8. Fluorescent inverted microscope equipped with UV light source.
9. Pipette controller: Two sets (left- and right-hand side).
10. Micromanipulator arms: Two sets (left- and right-hand side).
11. CellTram Air microinjector.
12. CellTram vario microinjector.
13. Materials for preparation of nucleus-donor cells: *See* Subheading 2.4.3.

2.5.2 Management of Reconstructed Eggs

1. H-BSA: 0.5% w/v of bovine serum albumin in HBSS.
2. Egg-holding media: Pure Chinook salmon ovarian fluid (CSOF). Sterilize with a 0.22 μm filter.
3. Embryo water: 10% of Hanks' balanced salt solution (HBSS).

3 Methods

3.1 Selection of Zebrafish Strains to Use

1. Selection of zebrafish strains used for supplying eggs: Use the F1 cross between AB and Tubingen (TU) strains, so-called TAB, as egg donors. Every 6 months, AB and TU pairs should be replaced with new ones to guarantee the quality of TAB fish. The first generation (F1) of TAB fish are the best at consistently supplying good-quality eggs, as measured by percentage of fertilized eggs that reach larvae/adult stages. When performing SCNT sessions twice or three times a week, at least 20 pairs of adults AB and Tubingen, between 6 and 10 months old, should be maintained in the laboratory. In summary, approximately 50 pairs of TAB are sufficient for a workload of SCNT three times weekly. TAB breeders, especially females, should be replaced every 3–4 months.
2. Select zebrafish strains used for supplying nucleus-donor cells. The strain of the donor nucleus will vary depending on the strain that we are looking to clone (*see* **Note 1**). For practical purposes, when first training for SCNT, we strongly

recommend using a transgenic line with a distinctive phenotype, or a mutant strain bearing a recessive gene that can be observed noninvasively. These nuclear donors will help determine the success or failure of SCNT much faster, i.e., in less than 24 h.

3. Source of zebrafish: The best sources for zebrafish are the national and regional resource centers such as Zebrafish International Resource Center—ZIRC (USA), the European Zebrafish Resource Center—EZRC, the China Zebrafish Resource Center, or the Japanese Zebrafish Stock Center at the Riken Institute. These centers provide certified specific pathogen-free animals. Alternatively, for a beginner, a transgenic line that expresses GFP under a muscle-specific *myosin light polypeptide 2* (*mylz2*) promoter, also known as Glofish® [5], can be easily purchased from a pet store, and used as long as pairs are quarantined, and only bleached, fertilized eggs are allowed to enter the fish room.

3.2 Fundamental Practices of Working with Embryos and Adult Zebrafish used for SCNT

3.2.1 Breeding Practice and Embryo Culture

Breeding fish is fairly simple, but there are important details that must be taken into account. Zebrafish is a year-round breeder. Females have asynchronous oogenesis, and can constantly supply oocytes/eggs [6]. A gravid female will display a distended abdomen. However, this must be differentiated from egg-bound syndrome, obesity, and a full stomach after feeding (*see* **Note 2**). Males have long-slender bodies, and are comparatively more active than females, and are bright, golden striped. General characteristics indicating animals in good health include optimal size at a specified age; sleekly bright color; alertness, i.e., proper response to light and sound; and anticipation of feeding (*see* **Note 3**).

1. Select male and female at approximately 3 h before the time of last meal of the day prior to breeding. Separate male and female in different containers and feed the fish.
2. Set a mating box, i.e., a small tank that keeps males and females separated by a mesh. After the fish have finished their meal, place them into the mating box but keep them isolated from each other using the mesh.
3. The following morning, as soon as the lights come on in the fish room, remove the mesh and let courtship behavior start (*see* **Note 4**).
4. Prepare 10 ppm of methylene blue in embryo medium. The solution is used for rinsing the fertilized eggs.
5. Collect fertilized eggs after 3 h has passed (*see* **Note 5**). Rinse embryos thoroughly in "embryo water" to clean out excrements, and then maintain in 10 ppm of methylene blue in embryo medium in 28.5 °C incubator. Alternatively, washing

the embryos with 0.05% solution of bleach in embryo medium is also an option.

6. Record the embryonic development and monitor the embryo every 3 h until entering gastrulation, and daily until hatching (*see* **Note 6**). The goal is to consistently produce a minimum of 90% of hatching embryos (72–48 h postfertilization) and to have at least 70% of live feeding fry at 2 weeks after fertilization. The type of food, feeding program, and environmental hygiene are critical factors for obtaining healthy embryos. Laboratory practices that yield such survival rates with fertilized embryos speak of proper husbandry practices, the key foundation for working with SCNT embryos (*see* **Note 7**).

3.2.2 Establish TAB Crossbreed

The recipient egg is crucial for the success of SCNT, and as such they should be of superior quality. Females used as egg donors need more attention to achieve excellent reproductive competency. We have found that F1 TAB (and not F2, F3, etc.) are the best (*see* **Note 8**). F1 TAB females usually inherit the most desirable features of TU and AB, including both high egg production and tolerance to micromanipulation and handling. TAB juveniles should be raised with the standard raising protocol to achieve optimal health.

1. Select adult fish of AB and Tubingen lines that have an optimal breeding efficiency (>90% consistency fertilization ratio).
2. Crossbreed AB and Tubingen by a conventional breeding practice as described in Subheading 3.2.1.
3. Collect fertilized eggs (TAB F1) and continue the cultivation process as described in Subheading 3.2.1.
4. Start the breeding adolescent TAB fish at 3 months old with a resting interval of 3 weeks between breeding.
5. When breeding for the first time, placing the fish in a group with a ratio of one male per three females is recommended. After successful mating, change the ratio to a 1:1 male to female.
6. Record the breeding performance of each female. The females will become familiar with the breeding routine by the time they reach reproductive maturity at the age of 5–6 months old.
7. Separate the pairs with a consistently high fertilization rate (>90%), which will be used for SCNT experiments.
8. Feed F1 TAB breeders three meals per day to maintain reproductive soundness. They will be used as an egg donor when they are between 6 and 10 months old (*see* **Note 9**).

3.3 In Vitro Fertilization and Utilization of Egg-Holding Media

3.3.1 Gamete Collection

1. Prepare a stock solution of tricaine (3-amino benzoic acid ethyl ester, MS222) in a concentration of 0.4% w/v in embryo medium (*see* **Note 10**).
2. Prepare tricaine working solution by adding 3 mL of stock solution to 60 mL of fish water for a final concentration of 0.02% w/v.
3. Set up a 35 mm petri dish containing 5 mL of egg-holding media, a folded Kimwipe™ tissue paper, and a smooth-tipped glass rod.
4. Place male and female together by removing the mesh.
5. After mating behavior is observed in the breeding tanks, transfer the female into the sedating solution and wait for approximately 2 min or when the fish stops movement and begins to breathe slowly.
6. Transfer the fish to a beaker filled with fish water for a few seconds to remove any remaining MS222. Place the female in a folded Kimwipe paper with its belly-side up exposing the genital opening, and dry the genital area with another clean Kimwipe™ paper (*see* **Note 11**).
7. Gently hold the fish in both flanks using the thumb and a middle finger. Using a curved glass rod, apply a light pressure in the abdomen, rostral from the genital opening. Mature eggs arrested in metaphase of meiosis II (MII eggs) are then released directly into a petri dish containing 1 mL of egg-holding media.
8. Apply the same sedation protocol to the male. Use a cosmetic sponge with sizable groove to handle the male instead of the paper and fingers. The male is stabilized in the slit of a sponge in supine position.
9. Dry the genital area and adjacent fins with a Kimwipe paper prior to sperm extraction.
10. Gently press one flank of the fish with a ring finger tip while pressing the opposite side with a smooth glass rod. The milt will be released and instantly absorbed into 5 μL glass pipette by a capillary force.
11. Transfer the milt to a 1.5 mL microtube containing 50 μL of chilled Hanks' balanced salt solution (HBSS).
12. Assess the sperm quality such as sperm motility by adding small amount of rearing water or embryo medium to the milt as described in "The Zebrafish Book" [7] (*see* **Note 12**). Good sperm start to swim progressively (>90%) and then the movement ceases and stops after approximately 2 min (*see* **Note 13**).

3.3.2 In Vitro Fertilization (IVF)

IVF is useful for evaluating the developmental potential of the recipient eggs after the SCNT session has ended. IVF is also used to examine the quality of Chinook salmon ovarian fluid (CSOF), which in our hands is the best holding media for unfertilized MII eggs [2]. Specific protocols for zebrafish IVF have been described in The Zebrafish Book [8] and more recently optimized by Matthews and colleagues [9]. Here, we describe the IVF protocol that is currently used in our laboratory (*see* **Note 14**).

1. Collect eggs and sperm by the described method of Subheading 3.3.1. Inactivated eggs are kept in holding media in a moist chamber. Keep the sperm suspension (diluted in HBSS) on wet ice at 5 °C until use (*see* **Note 15**).
2. Remove the inactivated eggs from CSOF and quickly rinse the eggs with 0.5% H-BSA.
3. Transfer the rinsed eggs into a 60 mm petri with a minimal amount of 0.5% H-BSA.
4. Deliver 50 μL of sperm suspension on top of the egg cluster, and simultaneously add 1 mL of embryo medium.
5. Incubate the eggs for 2 min at room temperature, and then add the additional 8 mL of embryo medium.
6. Place the petri dish with the fertilized embryos in the incubator at 28.5 °C and avoid any disturbance.
7. Observe the eggs, and remove unfertilized and dead embryos at 3, 6, 24, and 48 h postfertilization.

3.3.3 Preparation of Egg-Holding Media

At the time of ovulation, the mature egg is arrested in metaphase II of meiosis (MII). Once it is released to a spawning medium, egg activation is triggered spontaneously upon exposure to hypotonic water. Holding media is important for sustaining the eggs in an inactivated stage, maintaining its highest MPF activity possible [10]. Collection of inactivated eggs from a fertile female, and preserving their metaphase-arrested stage, is critical for the successful outcome of SCNT. Activation of zebrafish eggs can be artificially blocked for a short period using a physiological buffer or a biological fluid. Hanks' balanced salt solution supplemented with 0.5% bovine serum albumin (0.5% H-BSA) can preserve the MPF activity of inactivated egg for 30–60 min [10]. Coelomic fluid (ovarian fluid) of gravid female from several strains of Salmonidae has the ability to maintain zebrafish eggs at an inactivated stage for extended period (*see* **Note 16**). Zebrafish eggs can be kept in ovarian fluid of coho salmon for 1.5 h [11], rainbow trout for 2 h (unpublished data), and Chinook salmon for 5 h [10] (*see* **Note 17**). We recommend using Chinook salmon ovarian fluid (CSOF) which allows us at least 4 h to work with the eggs throughout the SCNT procedure.

1. Obtain the coelomic fluid by squeezing unfertilized eggs from a healthy gravid Chinook salmon female and straining off the fluid surrounding the eggs.
2. Inspect the fluid from each female individually. The fluid must be clear to slightly yellow in color. It should be discarded when contaminated with blood or excrement.
3. Remove insoluble particles or sediments of CSOF by centrifugation at 200 RCF for 15 min at 4 °C, and then aliquoting and subsequent storage at −80 °C until use.
4. At the time of use, CSOF is thawed and filtered using a 0.44 μm filter followed by a 0.22 μm filter.
5. 5. Perform IVF to test the quality of CSOF in every single batch. Use male and female zebrafish pairs that have been shown to be reproductively sound in previous breeding, and isolate sperm and eggs from them. Use as control a batch of CSOF that has been previously shown to be of great quality for keeping eggs at MII.
6. 6. Use approximately 200 eggs per test. Place half of the eggs in the CSOF to be tested, and the other half in control CSOF, and keep them at room temperature in a humidified chamber.
7. Every hour, until 4 h after egg retrieval, fertilize 30–50 eggs using the IVF technique described (*see* **Note 18**).
8. Record the fertilization rates, and compare it to the control (*see* **Note 19**).
9. A proper egg-holding media (CSOF) should be able to keep eggs inactivated for at least 4 h, and upon fertilization 70% of the eggs should be successfully fertilized (*see* **Note 20**).
10. Potentially, ovarian fluid from other types of Salmonidae could be used as an alternative to CSOF to keep zebrafish eggs arrested at MII. Each new batch of CSOF must be tested for its quality prior to use.

3.4 Essential Procedures for SCNT

3.4.1 Preparation for Micromanipulation

Our current technique for micromanipulation of an inactivated egg uses cooperative movements of a holder pipette, a supporting pipette, and an injecting needle. The holder pipette and supporting pipette are made using borosilicate capillary glass tube. The injecting needle is purchased from a commercially available needle that is used for intracytoplasmic sperm injection (ICSI). Select the inner diameter of the ICSI needle according to the size of the nucleus-donor cell (*see* **Note 21**).

1. The rest of the glass pipettes used during micromanipulation are pulled in the laboratory using a pipette puller.
2. To make a holder pipette, cut the tip off of a pulled glass pipette using a diamond cutter. Polish the tip with fine (P400) and

extra fine (P2000) sand paper to an outer diameter of 500 μm, and then fire-polish. Bend the holder pipette by heating the shaft, approximately 8 mm from the tip, until it reaches approximately a 30-degree angle.

3. To make a supporting pipette, cut the tip of a pulled pipette with a Microforge to an outside diameter of 20 microns. Then, melt the tip to remove any sharp edges.
4. To prepare the manipulation dish, place a rectangular shaped drop of 100 μL 5% polyvinylpyrrolidone (PVP) in CSOF (~100 μL) at the center of a 9 cm petri dish (*see* **Note 22**). Set a 10 μL drop containing donor cells in 1% PVP in DMEM adjacent to the corner of the rectangle (*see* **Note 23**).
5. After the drops are in place, slowly add mineral oil onto the dish to completely cover the surface of drops to prevent evaporation.
6. Set up a fluorescent inverted microscope equipped with a UV filter to visualize the DNA stained with Hoechst 33342 and a foot paddle that controls the UV shutter.
7. Install the micromanipulation setting (pipettes and pipette controllers) to the microscope including two sets of manipulators on the left- and right-hand sides. Place the holder pipette used for stabilizing the egg during the injection perpendicularly on the left micromanipulator arm, and connect it to a CellTram air microinjector. On the right side, fill an injecting needle completely with Fluorinert and connect it to a CellTram vario microinjector. A double pipette holder is required for setting up both the injecting needle and a parallel supporting pipette on the right micromanipulation arm.
8. Using left and right micromanipulators, practice the movement of both hands to control the holder, supporting, and transfer needles that will direct the orientation of an egg and inject the donor cell nucleus.
9. Practice firing the laser, and opening and closing the UV shutter with their respective foot switches (*see* **Note 24**).

3.4.2 Enucleation Practice

In our hands, laser ablation is the most convenient way to inactivate the genomic DNA of the egg or "enucleate" it. This technique does not require chorion removal nor egg aspiration [1], steps that had higher levels of technical difficulty. We recommend using a laser objective lens (40×, Hamilton Thorne©) that is compatible with the lens-mounting wheel of most inverted microscopes.

1. Set the laser power at 100% with a single laser pulse of 500 μs.
2. Using a Hoechst stock solution of 50 mg/mL in HBSS, prepare the working solution by diluting it 1000-fold in CSOF,

and vortex at full speed for one full minute to thoroughly dissolve the Hoechst.

3. Collect inactivated eggs using the conventional method of gamete collection (*see* Subheading 3.3.1), and then preserve the eggs in egg-holding medium.
4. Place the eggs in Hoechst staining solution (50 μg/mL of Hoechst) at 28.5 °C for 30 min, and keep the container in humidified chamber (*see* **Note 25**).
5. Rinse the eggs in fresh CSOF, and transfer them to a 30 mL petri dish containing 5% PVP in CSOF, five eggs at a time, and rinse them once prior to placing them into the manipulating drop (5% PVP in CSOF).
6. Micromanipulate one egg at a time in the following sequential steps:
   - Position the egg, using holder pipette and supporting pipette, until the micropyle is facing down, at the closest distance to the objective lens, i.e., bottom of the dish.
   - Stabilize the egg using negative pressure in the holder pipette. The metaphase plate is located in the animal pole, in the vicinity of the micropyle, generally adjacent to the first polar body.
   - Upon UV excitation of DNA stained with Hoechst®33,342, the bright blue color of the metaphase plate (asterisk-like structure) and the first polar body (round-compact structure) should be clearly visible (*see* **Note 26**).
   - Perform fine movements with your manipulator to have the egg DNA (metaphase) aligned with the laser-projected target.
   - Fire the laser at the metaphase plate using the foot switch twice (*see* **Note 27**).
7. During the training session for a new operator, perform IVF in all eggs that were enucleated, and observe their development. If the egg's DNA was properly destroyed, haploid embryos should be generated with the specific phenotypic signs [7] such as deformed head, short trunk, and pericardial enlargement (*see* **Note 28**).

3.4.3 Egg Quality Assessment

The recipient egg plays a key role in the success of cloning. It contains the molecules needed to shut down the expression pattern of somatic genes and turn on the embryonic ones. MII eggs are obtained from a female that has previously been shown to be fertile by natural mating, and that have displayed proper mating behavior on the day of egg retrieval. At present, there are no objective methods to determine the best eggs for cloning or IVF for that

matter. However, we have found morphological characteristics in eggs that correlate with successful SCNT (*see* **Note 29**).

1. Examine egg clutches for these characteristics:
   - Size: A zebrafish mature egg has the diameter of 730–800 μm on average [12, 13].
   - Microscopic structure (observation using bright field): The yolk of eggs with the best developmental potential is golden, and contains fine granules that are homogenous in size and distribution throughout the cytosol. The cortical granule vesicles (CGs) can be *see*n uniformly aligned at the animal pole, which can be *see*n as a clear, thin line at the cytoplasmic rim.
   - Uniformity of all eggs from a clutch is important. Nonviable eggs—either for IVF or SCNT—Usually come from a clutch that shows eggs of different sizes, with a nonhomogeneous cytosol, and lacking or displaying a partially developed micropyle.
   - Prematurely activated eggs are undesirable as well, since their levels of MPF begin to decline, decreasing the window of time in which the somatic cell DNA can be exposed to the egg cytosol [10]. Activated eggs are easily identified by looking at the shape of the chorion and the animal pole (*see* **Note 30**). When CG are released, the chorion separates from the egg's membrane, and the animal pole begins to form due to actin-mediated cytoskeletal rearrangement [14, 15].
   - Length of time that the eggs can be kept at MII-arrested stage. When using quality-tested CSOF, eggs collected from mature TAB female that displays good breeding performance can be maintained in CSOF without showing any morphological change for at least 4 h post-stripping.
   - Do not use egg clutches that have other overt signs of low quality such as chorion rupture; lysis of the egg cytosol; dark-khaki color of the yolk; and contamination with blood or excrement.
   - Aged eggs that have been kept in holding media for more than 4 h after the collection begin to display changes in the yolk, from fine granular texture to homogenously coarse, and the animal pole loses its yellowish glow and becomes opaque. Aged eggs should not be used for SCNT. According to a visual estimation, the ratio of cross-sectional area of yolk and cytoplasm (or vegetal vs. animal pole) shifts from 9:1 to be 6:4 as the egg ages.

3.4.4 Preparation of Nucleus-Donor Cells

The choices of nucleus-donor cells will vary depending on the objective of the experiment. Freshly isolated cells can be obtained from embryos at the 20- to 24-somite stages (*see* **Note 31**). For a specific cell type, transgenic lines that express tissue-specific reporter genes such as GFP are recommended. At present, there is a plethora of reporter gene lines for many tissue-specific genes to choose from. For zebrafish SCNT, we have used cells from heart, skin, and notochord [3, 16]. We have also used somatic cells isolated from fertilized embryos that were cultured, expanded in vitro, frozen, and thawed (*manuscript in preparation*). Regardless of the type of cells used, at least 100 single cells are needed for each person doing SCNT in one session (~4 h) (*see* **Note 32**). During the aspiration of the somatic cell into the injecting needle, the plasma membrane of the cell is broken, facilitating a quick exposure of the nucleus to the egg's cytosol. The cell membrane of some cells is more easily broken than others, depending on the tissue of origin. The size of donor cell will determine the internal diameter of the injecting needle. The method of cell dissociation used to obtain a single-cell suspension depends on the source of cells: fresh embryonic tissue, cultured cells, or specific cell types:

1. **Steps 1–5** for freshly isolated embryonic cells: Prepare embryos at 20- to 24-somite stages by performing natural fertilization or IVF on the day prior to SCNT experiments.
2. Remove chorion with fine-tip forceps, and then rinse the dechorionated embryo in a drop of 1% antibiotic in LHC media; repeat the process three times.
3. Cut the embryonic tail bud of the embryos and chop them into fine pieces with a surgical blade (*see* **Note 33**). Transfer the tail pieces into a 1.5 mL microtube.
4. Perform the mechanical dissociation by pipetting up and down 30× to release single cells (*see* **Note 34**).
5. Add an equal volume of 2% PVP in serum-free DMEM to the cell suspension to make a final 1% PVP cell suspension. Transfer the cell suspension into a drop of 1% PVP-DMEM previously placed in the manipulation dish (*see* Subheading 3.4.1).
6. **Steps 6–10** for freshly isolated embryonic cells: Use of cultured cells removed from 1 well of a 6-well plate. Discard the culture media from the well, and rinse the well with PBS 3×.
7. Add 200 μL of low concentration of trypsin-EDTA (0.025%) or TrypLE-Express and incubate the plate at 28.5 °C for 5–10 min or until cell detachment is observed.
8. Add 1 mL of 10% fetal bovine serum (FBS) in DMEM into the well, then transfer the cell suspension to 1.5 mL tube, and centrifuge at 700 RCF for 5 min.

9. Remove the supernatant and resuspend the cell pellet with 200 μL serum-free DMEM.
10. Add an equal volume of 2% PVP in DMEM (200 μL) to the cell suspension to make 1% PVP cell suspension and transfer the cell suspension into a drop of 1% PVP-DMEM previously placed in the manipulation dish (*see* Subheading 3.4.1).
11. **Steps 10–12** for specific cell types: Occasionally an additional step is required such as a final selection of cells expressing a specific reporter gene using UV light illumination.
12. In case of zebrafish erythrocytes, a NaCl solution must be added into the cell suspension to a total of 0.3% (v/v) as well as 1 mg/mL EDTA to make the red blood cells swell and become more fragile.

3.5 Workflow Process for SCNT and Egg Post-Activation Management

3.5.1 Microinjection of the Somatic Cell Nucleus Through the Micropyle

Under normal circumstances the micropyle serves as the entry point for fertilizing spermatozoa. It is located in the animal pole. It has a cone shape and is composed of the micropylar groove (~8 μm in diameter) and the micropylar pit (3 μm in diameter) [17]. The external opening of micropyle is slightly larger than 8 μm, reaching its narrowest diameter at the end closest to the egg membrane. After the enucleation process has been performed, the nucleus-donor cell is immediately transplanted into the egg. The three-dimensional movement of the injection needle is controlled by the right micromanipulator, and the aspiration and injection of the somatic cell are controlled by a microinjector. We recommend having the microinjector filled with mineral oil and the microinjection needle with Fluorinert. Once the needle is immersed into the manipulation drop (5% PVP in CSOF) it will aspirate some of it. The microinjection needle will then have three different solutions: closest to the microinjector is mineral oil, followed by Fluorinert in the middle, and manipulation media at the tip of the needle (*see* **Note 35**). We recommend having the meniscus between the Fluorinert and the manipulation media within the microscope field of view. If at any time during the manipulation process the meniscus moves on its own, without rotating the microinjection knob in any direction, we must stop and check for the presence of air bubbles anywhere along the tubing or the needle (*see* **Note 36**). Cells that have approximately the same diameter as the inner diameter of the needle are then aspirated one at a time. When the cell is aspirated, the plasma membrane will break, an event that can be easily visualized. The cell loses its original shape, and remains inside the needle as small debris and a nucleus.

1. Handle the egg and perform enucleation procedure using the technique described in the step in Subheading 3.4.2. Rotation of the microinjector knob counterclockwise or clockwise is

used for aspiration or release of fluid—respectively—in both the holding and the microinjection needles.

2. Locate the animal pole (*see* **Note 37**) and place its widest point at 3 o'clock in the field of view, where the cytoplasm is most visible (*see* **Note 38**).
3. Move the injecting needle into the drop where the nucleus-donor cell suspension is located (*see* **Note 39**), and aspirate a cell into the needle by the technique described in Subheading 3.4.4. When experiencing difficulties breaking the cell, aspiration and release of the cell can be repeated.
4. Reposition the egg to allow the micropyle to be perpendicular to the injecting needle.
5. Align the tip of the injecting needle to be at the same level as the micropyle by carefully focusing the microscope to allow a sharp view of the micropylar groove first, and then the injecting needle is adjusted up or down until its tip is also sharply in focus.
6. Gently advance the injecting needle through the micropyle opening, gliding along the groove and the pit, until it pierces through the eggs' cytoplasm.
7. Transfer the cell's nucleus, along with the remaining parts of the cell cytosol into the egg's animal pole, and then slowly withdraw the injection needle (*see* **Note 40**).
8. After releasing the nucleus along with the cell debris into the egg cytosol, place the eggs in 100% CSOF (without PVP) until activation.
9. Cell debris often causes clogging of the needle. Use a clean drop of cell-free 1% PVP in CSOF, that is placed on the same manipulation dish, to wash the needle by repeatedly aspirating and releasing the solution to clear out the debris.

3.5.2 Management of the Reconstructed Eggs

Unfertilized MII eggs have a soft chorion, and are much softer than an egg that has been recently fertilized. Unfertilized eggs are more susceptible to damage during micromanipulation; these include UV exposure, laser enucleation, and microinjection of the nucleus.

1. After the nucleus is injected into the egg cytosol, the reconstructed eggs are transferred to a 35 mm dish containing 2 mL of plain CSOF for 15-min equilibration (*see* **Note 41**).
2. Rinse the reconstructed eggs in 0.5% H-BSA to wash off the CSOF.
3. Transfer the reconstructed eggs to room-temperature embryo water in a 9 cm petri dish and immediately move the cultivating dish to an incubator at 28.5 °C.

4. If the eggs are placed in embryo water as a group, activation must be monitored after 5–10 min passes; those that fail to activate or show signs of degeneration must be removed from the group (*see* **Note 42**).
5. After 15 min in embryo water, properly handled eggs must resemble fertilized embryos of the same age.
6. Observe the cell division that should start 40 min after activation, and following the stages of embryonic development as if the SCNT eggs were fertilized embryos (*see* **Note 43**). Development must be monitored continually, at least every 2 h during the first 8 h after activation.
7. Raise cloned embryos using the same protocols used for rearing normal zebrafish embryos. Change 50% of the embryo water every morning and evening to prevent potential contamination (*see* **Note 44**).
8. Cloned fry can be moved to a zebrafish-housing system that has a slow rate of water circulation when they are 10 days old.
9. Cloned zebrafish should be individually raised until genetic testing is performed. Once it is confirmed that animals have the same genome, they can be housed in the same tank.

3.6 Trainee Training Schedule for Learning Zebrafish Cloning

We present a training schedule for the trainees (Table 1) that has proven to work in our laboratory.

3.6.1 Step One (1–1.5 Months)

During this first period the operator learns basic zebrafish husbandry and the fundamental practices of micromanipulation. These include the following:

1. Natural breeding for the generation of TAB fish, including selection of male and females, setting breeding tanks, selection and handling of fertilized embryos, and raising of the fry.
2. The preparation of micromanipulation needles should start simultaneously with basic husbandry and breeding techniques. Detailed instructions on how to make these tools are described elsewhere [4].

3.6.2 Step Two (2 Months)

In this session the operator will learn IVF, preparation of egg-holding media, and advanced manipulations. Specifically:

1. Master the IVF technique (*see* **Note 45**):
   - Proper use of sedation of males and females for gamete collection using tricaine. Training must include specific signs of the drug effectiveness taking into account that sensitivity varies among individuals.
   - Gamete collection and fertilization (described in Subheading 3.3).

Table 1
A training timetable for practicing of the preparation process and mandatory techniques of zebrafish somatic cell nuclear transfer

Training timetable			
Basic training (1–1.5 months)	**Advanced techniques (2 months)**	**Executing all SCNT steps (2–3 months)**	**Master all skills**
Supplying adults fish 1. AB and Tubingen strain 2. Pairs of transgenic line	*TAB breeding training* 1. Sorting male and female AB-F1 maintaining a ratio of 1:3 (male:female) 2. At 3 months of age, breed the fish as groups in tank with 1:2 ratio (male:female) 3. Record the breeding performance	*Exam the egg quality of TAB female* 1. Breed the TAB-F1 in a tank with 1:1 ratio and record the performance 2. Record % fertilization of individual females	*SCNT simulation* 1. Set up breeding pairs in the afternoon of a day before SCNT 2. Breed the fish in the morning and separate the female after the first one or two mating behaviors and before spooning
Practicing the breeding technique 1. Breeder selection 2. Mating behavior observation 3. Collection and culture of fish embryos		*Preparation of nucleus-donor cells* 1. Isolation of embryonic fibroblast: Tail tip, etc.	3. Prepare all reagents 4. Prepare nucleus-donor cells 5. Set up manipulation dish, pipettes, and needle
Establishing TAB-F1 line Crossbreeding of AB and Tubingen and keeping breeding pairs that demonstrate high fertilization rate and hatching rate (>90%)	*In vitro fertilization (IVF)* 1. Fish anesthesia 2. Egg collection 3. Sperm collection	*Manipulation practice* 1. Microinjection via a micropyle 2. Practice by injecting cell culture media such as LHC without adding animal serum 3. Management of reconstructed egg	6. Check the % of fertilization from natural breeding 7. Collecting eggs 8. Eggs' Hoechst staining 9. Start the process of enucleation and cell transplantation 10. Management of reconstruction eggs 11. Monitoring development of clones
First steps of micromanipulation 1. Operation of the manipulator and UV-equipped microscope 2. Manipulation setup 3. Making the pipettes	*Preparation of egg-holding media* 1. Acquiring salmon ovarian fluid 2. Quality control of CSOF by IVF *Manipulation practice* 1. Consistently holding inactivated eggs with small aspiration pressure by the holding needle 2. Aligning of the inactivated egg in any position with supporting pipette and injecting needle 3. Hoechst staining and visualization of the micropyle		

(continued)

Table 1
(continued)

Training timetable			
Basic training (1–1.5 months)	**Advanced techniques (2 months)**	**Executing all SCNT steps (2–3 months)**	**Master all skills**
	Enucleation practice 1. Hoechst staining 2. Laser ablation 3. Exam viability of the enucleated egg by IVF and record % of haploid embryo production		

2. Testing of different batches of CSOF for their capacity to maintain the fertility of unfertilized eggs for several hours: CSOF is usually collected once a year, and it is stored at −80 C in multiple batches. The capacity of each batch of CSOF to keep eggs fertile varies; therefore IVF is used to test whether a batch of CSOF can keep eggs viable for at least 4 h (described in Subheading 3.3).
3. Practice laser-assisted enucleation of MII eggs to master fine coordinating movements of both hands and both feet, three times a week (*see* **Note 46**). At the end of step two, the trainee should be capable of gently moving a MII egg in a three-dimensional space; identifying the egg's DNA; performing laser-assisted enucleation; functional verification of enucleation; and identifying the egg's micropyle.

3.6.3 Step Three (Anywhere from 3 to 6 Months Depending on the Dexterity of the Trainee)

The final training step is executing all steps of SCNT. At the end of this training period the trainee should be able to perform the following procedures effortlessly:

1. Determine the quality of mature inactivated eggs from different females using morphology of the eggs and validation using IVF (described in Subheading 3.4.3).
2. Prepare single-cell suspensions of donor cells (described in Subheading 3.4.4).
3. Perform egg microinjections through the micropyle (described in Subheading 3.5.1).
4. Perform the egg activation process and raising of the cloned fish (described in Subheading 3.5.2).

3.7 Clone Identification

Phenotypic assessment is useful when the nuclear donor has a distinct characteristic from that of the egg donor. Genotypically, cloned fish must be proven identical to the animal donor of the nucleus, but not to the egg donor. Mitochondrial DNA must match that of the egg's donor. Determination of proper ploidy of the clones is also recommended.

1. Phenotypic identification of cloned animals can be easily accomplished by using golden or fluorescent-labeled zebrafish strains.
2. Ploidy analysis can be done using flow cytometry and/or karyotyping by R-banding [18].
3. DNA genotyping can be performed using 11 single-nucleotide polymorphism (SNP) markers previously described in great detail [2]. The SNP-based genotyping can be done using PCR and restriction fragment length polymorphism (PCR-RFLP). Final SNP sequencing is recommended.

4 Notes

1. In our laboratory, we have been able to clone fish from Tubingen, AB, Tubingen long-fin, SAT, and Golden strains.
2. Egg-bound syndrome is a phenomenon observed when females accumulate eggs in their abdomen without ovulation.
3. Egg donors must also be free of body abnormalities and overt clinical symptoms of disease. Experienced personnel should be able to identify proper animals for breeding.
4. At times, aggressive behavior is displayed by either males or females; therefore, it is advisable to place a plastic plant or some crumpled mesh inside the tank to provide cover and prevent injuries of submissive animals.
5. Proper husbandry practices will ensure that females release all mature eggs from their belly, decreasing the potential of developing egg-bound syndrome.
6. The use of a stereomicroscope to evaluate development is required to remove embryos that develop abnormally.
7. Methods and procedures for embryo culture are described in The Zebrafish Book [7].
8. AB females are highly reliable at producing eggs consistently, but their eggs are not sturdy enough for micromanipulation. Tubingen females, in our hands, are more difficult to breed than AB females, but their eggs are sturdier and more suitable for micromanipulation.

9. At the time of this writing, it was announced that the TAB zebrafish strain was to be phased out at the ZIRC. The replacement strain, NHGRI-1, is also derived from a closely related TAB line and reportedly more resilient. Until we run comparisons between NHGRI-1 and TAB F1 females on their capacity to produce eggs that can be used for SCNT, we recommend that new users make their own TAB-F1 fish directly from AB and TU crosses.
10. It is important to prepare all the materials prior to sedation of the fish, and to be fast as the fish should not be out of water for more than 2 min.
11. It is important to dry the genital area with another Kimwipe™ paper prior to harvesting the eggs in order to prevent unintentional activation of the eggs due to water exposure.
12. For example, 1 μL of sperm and 10 μL of water.
13. Zebrafish sperm can be preserved in an inactive stage in H-BSS for up to 1.5 h at 5 °C.
14. All the materials to be used must be prepared beforehand to allow for a smooth workflow including 0.5% bovine serum albumin in HBSS (H-BSA) at room temperature; embryo medium at 28.5 °C; and a 60 mm petri dish.
15. If sperm is used after more than 1 h of being held at 5 °C, we recommend rechecking their motility rate before adding it to the eggs.
16. The molecular mechanism by which ovarian fluid is capable of maintaining zebrafish eggs inactivated remains to be elucidated.
17. Different salmon breeds provide ovarian fluid with different holding potency.
18. Fresh sperm is used for each round of IVF, so that at least one male is squeezed every hour. Quality of milt is assessed prior to IVF.
19. Evaluation of the CSOF quality must be done in conjunction with a control fluid.
20. For the purpose of this protocol, successful fertilization means morphologically normal embryos as determined at 48 h postfertilization.
21. Due to the size of micropylar pit, ICSI needles with an inner diameter of less than 10 μm should be used.
22. The solution of 5% PVP in CSOF is used to maintain the collected eggs, which are arrested at metaphase II of meiosis (MII).
23. The relative position of the two drops (100 and 10 μL) is important. They must be placed in such a way that allows

pipettes to move freely, without accidentally merging the two drops.

24. The coordination of these movements is intimidating at first; however, with enough practice, the operator will find that the procedure becomes second nature, and muscle memory does most of the work. In turn, development of SCNT embryos increases, mostly due to the speed and flawless execution of the procedure; that is, the faster and gentler micromanipulation is done, the higher the chances of making a healthy clone.
25. DNA is labeled with 50 μg/mL of Hoechst (1 μL of Hoechst 33342 stock solution in 1 mL of CSOF) to locate the metaphase plate and the first polar body.
26. An effort should be made to minimize the egg's UV exposure; ideally, locating the metaphase should be accomplished using no more than 5 s of UV exposure. Excessive exposure of ultraviolet irradiation is harmful to the egg, and can cause denaturation of the chorion, leading to egg activation problems.
27. With the laser parameters we described, we have not experienced detrimental impact in the egg quality; however, the laser power and pulse duration must be adjusted for laser models different than Hamilton-Thorne.
28. Transgenic females as egg donors bearing a reporter gene, i.e., GFP or RFP, can also be used to determine proper ablation of the female DNA.
29. Quality evaluation is done immediately after the eggs are isolated from the female and placed in CSOF, and throughout the SCNT session that usually takes up to 4 h.
30. Morphological changes in unfertilized eggs help determine the degree of maturity of the eggs, and whether they have been prematurely activated.
31. Recommended for those who try to implement zebrafish SCNT in their laboratory for the first time.
32. We recommend preparing the donor cell's suspension prior to the egg collection, or alternatively while the eggs are being incubated in Hoechst staining solution.
33. Work under a stereomicroscope.
34. This method sidesteps the use of enzymes that must be followed by rinsing the cells using centrifugation.
35. To guarantee precise aspiration and injection of the cells, there should be no air spaces in between the three fluids.
36. If there are no bubbles but the meniscus continues to move, the O-rings in the microinjector's tubing must be replaced.

37. Locating the micropyle requires some familiarity with the egg's gross appearance through the microscope. In high-quality MII eggs, the animal pole of the egg, or where the cytosol is observed, is quite thin, and it can be difficult to find for an untrained eye.
38. This is the point where the micropyle can be most easily identified.
39. The donor cells are placed in a suspension of 1% PVP, which helps lubricate the inner wall of the needle.
40. In some cases, the micropyle is pulled backward in the same direction of receding needle. This should not present a problem as it can be gently pushed back into its position using the injection needle.
41. We found that this step helps maintain the developmental potential of the eggs, and we speculate that it may facilitate chromatin remodeling and overall genome reprogramming of the nucleus.
42. Alternatively, reconstructed embryos can be placed individually in a 96-well plate, in which case the rapid removal of abnormal embryos is not that critical.
43. We frequently encounter a delay of the first cycle of cleavage in reconstructed egg, likely due to a centriolar mediated karyokinesis defect [19].
44. Regardless of whether the embryos are cultured individually or as a group.
45. IVF is always used as an indicator of egg developmental potential and quality of the reagents used; therefore, mastering the technique is required.
46. This is a time to test the quality of the micromanipulation needles made during the first training session (Subheading 3.6.1).

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Chapter 23

An Accessible Protocol for the Generation of CRISPR-Cas9 Knockouts Using INDELs in Zebrafish

Cara E. Moravec and Francisco J. Pelegri

Abstract

The ability to create targeted mutations in specific genes, and therefore a loss-of-function condition, provides essential information about their endogenous functions during development and homeostasis. The discovery that CRISPR-Cas9 can target specific sequences according to base-pair complementarity and readily create knockouts in a desired gene has elevated the implementation of genetic analysis in numerous organisms. As CRISPR-Cas9 has become a powerful tool in a number of species, multiple methods for designing, creating, and screening editing efficiencies have been published, each of which has unique benefits. This chapter presents a cost-efficient, accessible protocol for creating knockout mutants in zebrafish using insertions/deletions (INDELS), from target site selection to mutant propagation, using basic laboratory supplies. The presented approach can be adapted to other systems, including any vertebrate species.

Key words Genome editing, CRISPR-Cas9, INDELS, Targeted mutations, Zebrafish

1 Introduction

The last decade has seen the invention of multiple genome editing technologies, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) [1–3]. These technologies have given researchers the ability to not only genetically engineer traditional model systems at a significantly increased pace but have expanded the genetic tractability of nontraditional model systems.

CRISPR-Cas9 has revolutionized genome editing in part due to increased efficiency but also due to the fact that, when compared to ZFNs and TALENs, the mechanism of CRISPR-Cas9 action allows for greater flexibility in target selection and gene editing options. ZFNs and TALENs bind to target DNA directly via protein-DNA interactions, requiring construction of individual targeting proteins. CRISPR-Cas9, on the other hand, is targeted to the

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_23,

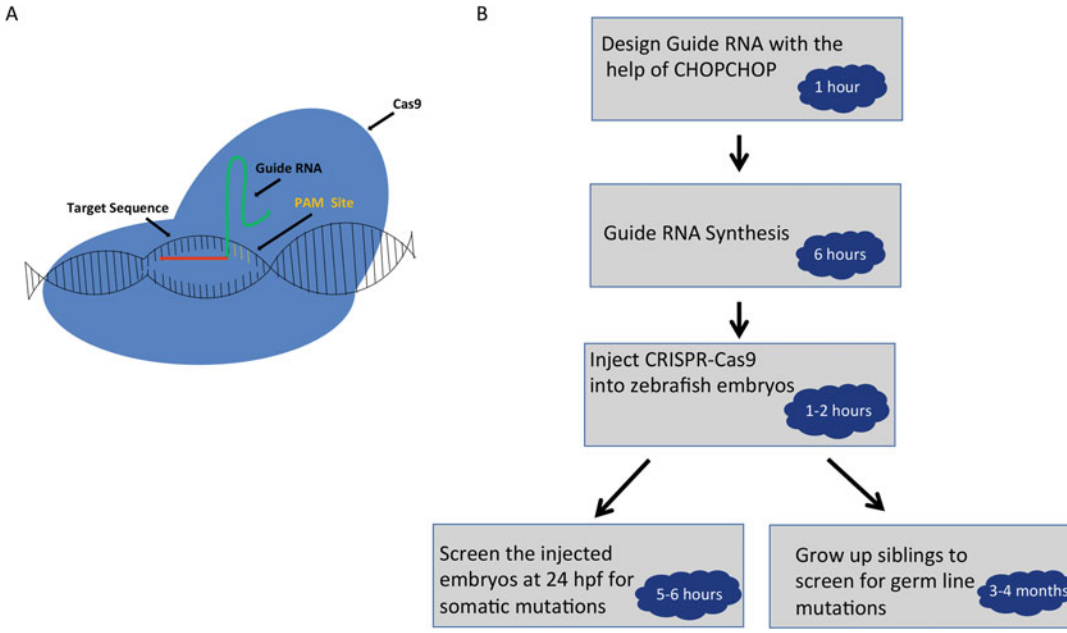


Fig. 1 The visualization of CRISPR-Cas9 in gene editing. (**a**) Diagram of CRISPR-Cas9. The Cas9 protein (blue) is used to cut DNA. It is directed to specific sites in the genome by the guide RNA (red) and the PAM site (yellow). The green hairpin of the guide RNA interacts with the Cas9. The Cas9 protein cleaves the DNA to promote DSB repair. (**b**) Visualization of the pipeline for the generation of CRISPR-Cas9 mutant line in zebrafish

DNA via a complementarity guide RNA and a requisite protospacer adjacent motif (PAM) sequence (Fig. 1a) [4].

Once bound to the DNA the method of DNA cleavage is also different. ZFNs and TALENs exploit the activity of a fused FokI endonuclease that requires homodimerization at the target site to cleave DNA [1, 2]. On the other hand, CRISPR-Cas9 employs the endogenous endonuclease domain found within the Cas9 protein [3], and does not require the binding of multiple constructs. These mechanistic differences allow CRISPR-Cas9 to have an increased number of targeting sites within the genome [5].

Similar to ZFNs and TALENs, CRISPR-Cas9 stimulates genome editing by creating double-strand breaks at targeted loci. The majority of the time when a double-strand break is created the cell will undergo nonhomologous end joining (NHEJ) by religating the DNA, thus creating insertions and/or deletions (INDELs) at the target site. These INDELs at target sites have the potential to create frameshift mutations and premature stop codons. These in turn allow analysis of the endogenous function of specific genes by examining the loss-of-function phenotype caused by the induced knockout mutations [1–3].

The use of CRISPR-Cas9 has transformed the field of cellular and developmental biology, with a continuing flow of new applications. In addition to the creation of knockout mutants, CRISPR-

Cas9 have been used for homology-directed repair to allow for knock-ins [6] and F0 genetic screens [7]. The catalytically inactive version of Cas9, which can bind the target sequence but does not induce DNA cleavage, has been used for gene regulation, epigenome editing, and chromatin imaging [8–11]. In addition, recent work has shown that other Cas proteins have the potential to edit DNA (Cas12a) and RNA (Cas13) [12, 13]. This chapter limits itself to present an efficient and user-friendly protocol addressing the creation of traditional INDEL knockouts using *Streptococcus pyogenes* Cas9, arguably of great value to the study of function and generation of models in any vertebrate model system. We use the zebrafish as an example model system but a similar approach can be readily applied to any other vertebrate species. With appropriate modifications, the methods presented in this chapter can also be adapted to the other applications of CRISPR-Cas9 and alternative Cas proteins. This protocol was compiled from aspects of several published articles [3, 14–16]. A workflow with approximate times is outlined in Fig. 1b.

2 Materials

2.1 Designing the Guide RNA (sgRNA)

1. ensembl.org
2. chopchop.org

2.2 Synthesis of Guide RNA

1. Constant oligonucleotide: (5′AAAAGCACCGACTCGGTGC CACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA CTTGCTATTTCTAGCTCTAAAAC 3′).
2. Target-specific oligonucleotide.
3. Nuclease-free water.
4. PCR plate/PCR tubes.
5. Thermal cycler.
6. T4 DNA polymerase.
7. 10 mM dNTPs.
8. PCR cleanup kit for elution into small volume.
9. MEGAshortscript T7 Transcription Kit (Invitrogen).
10. Tris/borate/EDTA buffer: 89 mM TRIS base, 89 mM boric acid, 2 mM EDTA.
11. 1.5% agarose gel solution: 1.5% agarose in Tris/borate/EDTA (TBE) buffer, with 0.25 μg/mL of ethidium bromide. Using a microwave, melt 1.5 g agarose in TBE. After the agarose is melted, add 2.5 μL of 10 mg/mL ethidium bromide. Allow the solution to cool down to touch and pour onto the mold for the gel apparatus.

12. Gel box and combs with approximately 0.625 cm wide wells.
13. 100% Ethanol.
14. 70% Ethanol: Mix 70 mL of 100% ethanol and 30 mL of nuclease-free water.
15. 1% Agarose gel solution: 1% agarose in Tris/borate/EDTA (TBE) buffer, with 0.25 μg/mL of ethidium bromide. Melt 1.0 g agarose in 100 mL TBE, and add 2.5 μL of 10 mg/mL ethidium bromide.

2.3 Injection of CRISPR-Cas9 into Zebrafish Embryos

1. Male and female zebrafish.
2. Breeding cages.
3. Standard injection equipment and supplies [17].
4. Cas9 protein (PNA Bio): Reconstitute lyophilized protein at 2 mg/μL in nuclease-free water. Store at −80 °C in 1 μL volume aliquots.
5. E3: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% methylene blue.

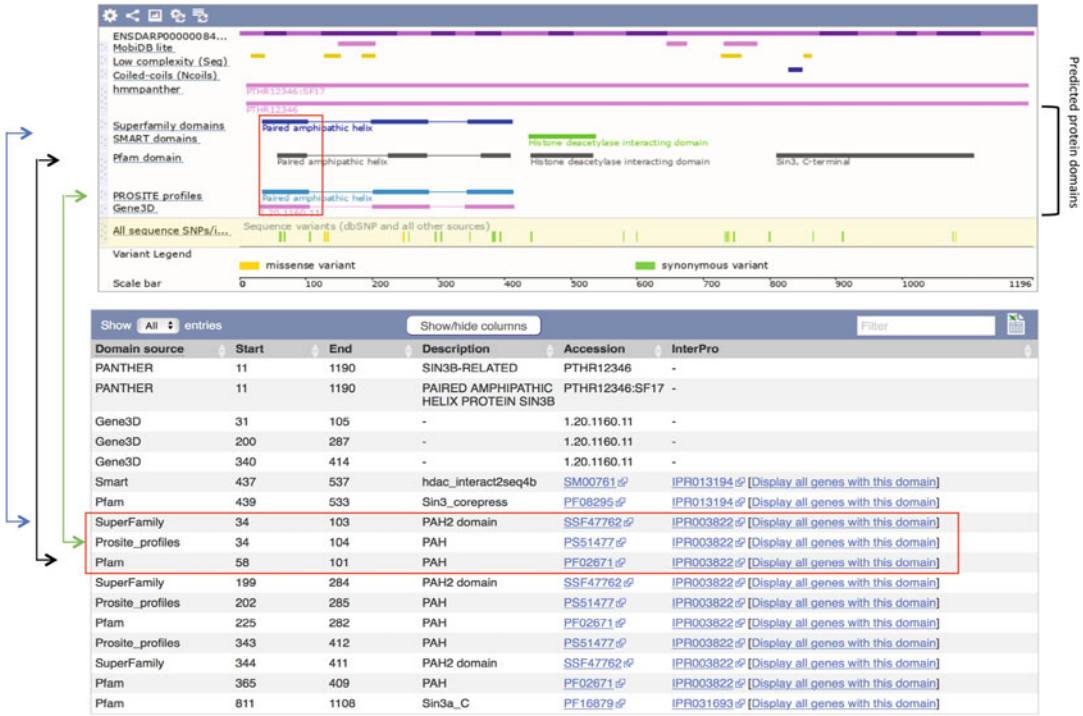
2.4 Screening Activity of the Guide RNA in Zebrafish

1. Primers to make a 100 bp fragment over the guide site, with the PAM site in the middle for genotyping.
2. Primers to make a 250 bp fragment over the guide site, with the PAM site in the middle for sequencing.
3. 2.5% Agarose gel solution: 2.5% Agarose in TBE, with 0.25 μg/mL ethidium bromide. In 100 mL TBE, melt 2.5 g agarose and 2.5 μL of 10 mg/mL ethidium bromide.
4. Gel box and combs that are 0.625 cm wide.
5. Tris/borate/EDTA buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA.
6. PCR reagents and materials.
7. DNA Clean and Concentrator: ZYMO Research.
8. 50 mM NaOH.
9. 1 M Tris–HCL ph 7.5.

3 Methods

3.1 Designing the Guide RNA (sgRNA)

1. After picking a gene of interest, use Ensembl.org to identify the domains of the protein. To prevent an alternative start codon the aim is to design the sgRNA in the first active domain of the protein (Fig. 2) (*see* **Note 1**).
2. Once the protein domain to be targeted has been identified, take the corresponding DNA sequence for that domain and enter it in FASTA format into CHOPCHOP (http://chopchop.cbu.uib.no) [14, 15] (*see* **Note 2**) (Fig. 3).



Domain source	Start	End	Description	Accession	InterPro
PANTHER	11	1190	SIN3B-RELATED	PTHR12346	-
PANTHER	11	1190	PAIRED AMPHIPATHIC HELIX PROTEIN SIN3B	PTHR12346:SF17	-
Gene3D	31	105	-	1.20.1160.11	-
Gene3D	200	287	-	1.20.1160.11	-
Gene3D	340	414	-	1.20.1160.11	-
Smart	437	537	hdac_interact2seq4b	SM00761	IPR013194 [Display all genes with this domain]
Pfam	439	533	Sin3_corepress	PF08295	IPR013194 [Display all genes with this domain]
SuperFamily	34	103	PAH2 domain	SSF47762	IPR003822 [Display all genes with this domain]
Prosite_profiles	34	104	PAH	PS51477	IPR003822 [Display all genes with this domain]
Pfam	58	101	PAH	PF02671	IPR003822 [Display all genes with this domain]
SuperFamily	199	284	PAH2 domain	SSF47762	IPR003822 [Display all genes with this domain]
Prosite_profiles	202	285	PAH	PS51477	IPR003822 [Display all genes with this domain]
Pfam	225	282	PAH	PF02671	IPR003822 [Display all genes with this domain]
Prosite_profiles	343	412	PAH	PS51477	IPR003822 [Display all genes with this domain]
SuperFamily	344	411	PAH2 domain	SSF47762	IPR003822 [Display all genes with this domain]
Pfam	365	409	PAH	PF02671	IPR003822 [Display all genes with this domain]
Pfam	811	1108	Sin3a_C	PF16879	IPR031693 [Display all genes with this domain]

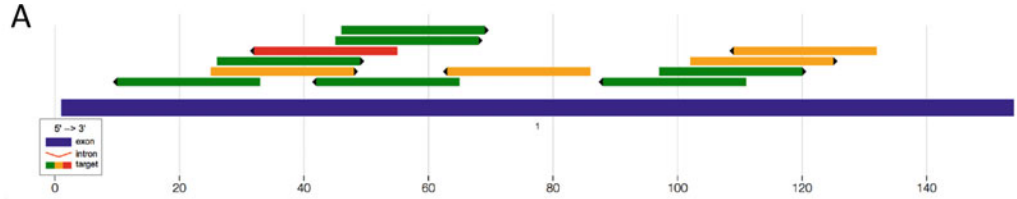
Fig. 2 An example of a domain page found on ensemble. The domain page for the Sin3b-predicted protein, found on Ensemble, showing that the first active domain (marked in red) is found from 34 to 104 amino acids. The location of the domain suggests that it is a strong candidate target to design a CRISPR against

3. Under the option setting "Cas9" go to 5′ requirement for sgRNA option and specify "GN" or "NG" (*see* **Note 3**). This option will allow for the use of a T7 promoter but will also provide increased targeting sites in the gene of interest over the traditional GG option. Then click "Find Target Sites!" (Fig. 3). The program will rank the available sgRNA options by taking into consideration the percentage of GC in the sgRNA, and ability for the sgRNA to self-complement and off-target effects of the sgRNA. These factors also are used to calculate the efficiency of the guide RNA (Fig. 3).
4. Pick any target that is predicted to have optimal efficiency (labeled green). Avoid predicted nonoptimal sgRNAs (labeled yellow and red). When you click the guide RNA of interest, the first 20 bases (red) of the guide RNA correspond to the spacer regions and should be included in the sgRNA, and the last three bases (NGG) correspond to the protospacer adjacent motif (PAM) site. Do not include the three bases corresponding to the PAM site in the design of the sgRNA (Fig. 4).
5. For each selected sgRNA, order a 60-base oligonucleotide that contains three components (*see* below): a T7 promoter (blue),

Fig. 3 An example of the CHOPCHOP webpage that helps design CRISPR targets. The protein domain of interest is entered in FASTA format in the target box. Once the target sequence has been entered, go to the options and change the 5′ requirements for sgRNA to GN or NG. Then hit "find target site." Adapted from CHOPCHOP.org, with permission

the target site from CHOPCHOP (red), and an overlap region (green). The latter overlap region will allow for annealing to the constant oligo (80 bp) that forms the constant hairpin structure recognized by Cas9, to fuse the hairpin sequence with that of the gene-specific oligo (*see* **Notes 3–5**) (Fig. 4b, c).

A



Please select one

Ranking	Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
							0	1	2	3	
1	GAGTTTCCCAGCTCTTCCATGGG	Sin3b:27	1	+	50	1	1	0	0	0	0.58
2	GGAATTTCAATCCGATACCCAGG	Sin3b:89	1	-	45	0	1	0	0	0	0.52
3	CGGATTGAAATTCCCAAGAATGG	Sin3b:98	1	+	40	0	1	0	0	0	0.51
4	GGACAAGGTCCGGGTGCCCATGG	Sin3b:43	1	-	70	1	1	0	0	0	0.49
5	GAAACTCTGTTGATAACTCCTGG	Sin3b:11	1	-	40	0	1	0	0	0	0.45
6	TGGGCACCCGGACCTTGTCCTGG	Sin3b:46	1	+	70	1	1	0	0	0	0.43
7	GGGCACCCGGACCTTGTCCTGGG	Sin3b:47	1	+	70	1	1	0	0	0	0.42
8	TGAAATTCCCAAGAATGGAATGG	Sin3b:103	1	+	35	1	1	0	0	0	0.57
9	AGAGTTTCCCAGCTCTTCCATGG	sin3b:26	1	+	50	0	1	0	0	2	0.46
10	GTAGAAATGCATTAAATCCCAGG	sin3b:64	1	-	35	0	1	0	0	1	0.56
11	AGAAATGCCATTCCATTCTTGGG	sin3b:110	1	-	35	0	1	0	0	1	0.38
12	CGGGTGCCCATGGAAGAGCTGGG	sin3b:33	1	-	65	0	1	0	1	2	0.53

B

#7
CATTGACACACCAGGAGTTATCAACAGAGTTTCCCAGCTCTTCCATGGGCACCCGGACCTTGTCCTGGGATTTAATGC
ATTTCTACCGCCTGGGTATCGGATTGAAATTCCCAAGAATGGAATGGCATTTCTTCAATCCCCATTTTCCTCGCAG

#1
CATTGACACACCAGGAGTTATCAACAGAGTTTCCCAGCTCTTCCATGGGCACCCGGACCTTGTCCTGGGATTTAATGC
ATTTCTACCGCCTGGGTATCGGATTGAAATTCCCAAGAATGGAATGGCATTTCTTCAATCCCCATTTTCCTCGCAG

C

#7 Gene specific part of sgRNA		
GGGCACCCGGACCTTGTCCTGGG		
Off-targets		
Genomic location	Number of mismatches	Sequence (including mismatches)
chr8:14198281	0	CCCAGGACAAGGTCCGGGTGCCC

Order this:

TAATACGACTCACTATA GGGCACCCGGACCTTGTCCT GTTTTAGAGCTAGAAATAGCAAG

D

#1 Gene specific part of sgRNA		
GAGTTTCCCAGCTCTTCCATGGG		
Off-targets		
Genomic location	Number of mismatches	Sequence (including mismatches)
chr8:14198301	0	CCCATGGAAGAGCTGGGAAACTC

Order this:

TAATACGACTCACTATA GGGTTTCCCAGCTCTTCCAT GTTTTAGAGCTAGAAATAGCAAG

Fig. 4 The potential guide RNAs for a specific site in a gene. (**a**) Output of the CHOPCHOP search, showing all the potential guide RNAs for the site of interest of Sin3b. The green targets are useful targets, while the yellow indicates use with caution and red targets are not recommended. (**b**) Genomic location of the two guide RNAs in the *sin3b* exon. (**c**–**d**) The gene-specific table from CHOPCHOP showing the guide site in red and the PAM site in blue. Below the table is the oligonucleotide to be ordered from an oligo synthesis source. Adapted from CHOPCHOP.org, with permission

Example of the gene-specific oligonucleotide with a T7 promoter

5' TAATACGACTCACTATA- N_{20} - GTTTTAGAGCTAGAAATAGCAAG 3'

6. If a T7 promoter is chosen and the spacer region is either NGN_{18} or GNN_{18}, the first N will need to be changed to a G to allow RNA synthesis (Fig. 4c).
For example:

5' TAATACGACTCACTATA- NGN_{18} -GTTTTAGAGCTAGAAATAGCAAG 3'

becomes

5'TAATACGACTCACTATA- GGN_{18} -GTTTTAGAGCTAGAAATAGCAAG 3'

3.2 Synthesis of Guide RNA

1. Assemble a mixture containing 10 μM of gene-specific oligonucleotide and 10 μM of constant oligonucleotide, and fill to 10 μL with nuclease-free water in a PCR tube (Fig. 5a).

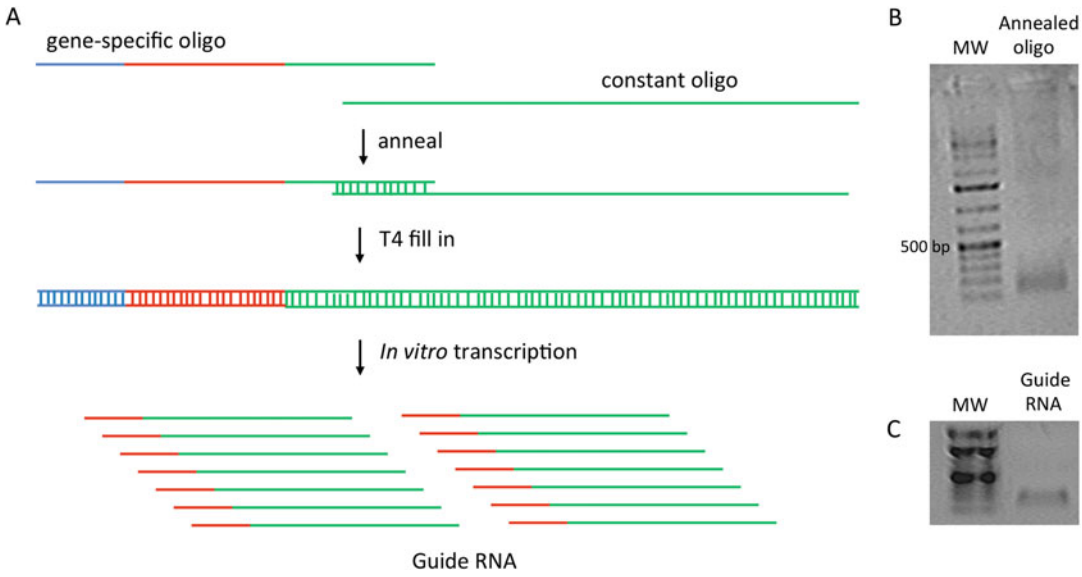


Fig. 5 Assembling of the guide RNA for CRISPR-Cas9? (**a**) A representation of how the annealing and T4 fill-in allow for the creation of guide RNA from oligonucleotides. (**b**) An example of what you should observe on a gel after the annealing and fill-in of the oligonucleotides. The dominant band should be about 120 bp. (**c**) An example of what you should observe on a gel when you run the guide RNA out after synthesis. You should observe one sharp band

2. Place the samples in the thermocycler and run a single cycle of the annealing program as shown below (Fig. 5a):

95 °C	5 min
95 °C → 85 °C	−2 °C/s
85 °C → 25 °C	−0.1 °C/s
4 °C	Hold

3. After the oligonucleotides have been annealed, use the fill-in activity of T4 polymerase to create a double-stranded fragment. Add 10ul of the following mixture to each sample: 25 mM of dNTPs, T4 DNA 10× polymerase buffer (e.g., NEB 2.1), and 15 units of T4 DNA polymerase (e.g., T4 NEB DNA polymerase), and fill to 10 μL with nuclease-free water. Incubate at 12 °C for 20 min (Fig. 5a).
4. Purify the template with a PCR cleanup kit for elution into small volume. Elute into 10 μL of nuclease-free water (*see* **Note 6**).
5. After purification run 2 μL on a 1.5% agarose gel to visualize a band around 120 bp (*see* **Note 7**) (Fig. 5b).
6. Transcribe sgRNA using the Ambion T7-MEGAshortscript kit (*see* **Note 8**). Assemble in 1.5 mL tubes and incubate for 3–4 h at 37 °C, as shown below (we use half-reactions, since the MEGAshortscript kit produces a high yield of RNA).

ATP	1 μL
GTP	1 μL
CTP	1 μL
UTP	1 μL
10× buffer	1 μL
T7 enzyme mix	1 μL
Template	1 μL–4 μL
Nuclease-free water	Up to 10 μL

After 3–4 h, add 1ul of TURBO DNase found in the kit and incubate for 30 min at 37 °C.

7. After synthesis, precipitate the resulting RNA using an ethanol/ammonium acetate protocol. To the 10 μL of RNA mixture, add 10 μL of ammonium acetate stop solution (included in the transcription kit) and 60 μL of 100% ethanol. Mix and incubate for 20 min at −80 °C. Spin in the microcentrifuge at maximum speed (minimum 13,000 × *g*) for 15 min at 4 °C.

Wash with 70% ethanol. Remove the supernatant and dry at room temperature for no more than 5 min. Resuspend in nuclease-free water (*see* **Note 9**).

8. Determine the RNA concentration with a nanodrop or spectrophotometer. Dilute the RNA stock solution in nuclease-free water to make a working dilution of around 1500 ng/μL ± 500 ng/μL.
9. Visualize the RNA quality on a 1% gel. Pour a 1% gel. While the gel is cooling, mix 1 μL of RNA with 1 μL of the loading dye that is found in the transcription kit. Run the mixture on a 1% gel for around 10 min. The RNA should be visible as a sharp band on the gel. If no RNA is observed on the gel repeat the RNA synthesis (**steps 1–8**) (Fig. 4c).
10. Once the RNA quality has been confirmed, store the RNA in 1 μL aliquots at −80 °C to prevent freeze-thaw cycles. If large quantities of RNA were synthesized a small amount of 1ul tubes can be created and the rest can be stored in larger volumes. Try to avoid more than three freeze-thaw cycles for larger volumes.

3.3 Injection of CRISPR-Cas9 into the Zebrafish Embryo

1. The night before the injection set up male and female zebrafish separated by a partition or insert in order to prevent overnight mating and limit breeding to the morning.
2. The morning of the injection, prepare the injection mixture in a 2:1 ratio of Cas9 protein:guide RNA. Typical amounts injected per an embryo are 400 pg Cas9 protein and 200 pg of guide RNA. If injecting multiple guide RNAs, the total RNA should equal 200 pg. The protein/guide RNA mix should be incubated at room temperature for 5 min allowing for the formation of the complex, and then stored on ice. In our hands, preparing about 5ul of solution allows injecting through the experiment without having to prepare more solution.
3. Combine the male and female zebrafish together and allow for breeding to obtain embryos. Collect embryos in 7–10-min intervals during breeding in order to obtain embryos born at a similar time, and record the time of fertilization.
4. Load the protein/guide RNA mix into the needle and microinject into one-cell embryos using the standard zebrafish injection protocol [17]. Aim to inject 1 nL into the blastomere itself (blastodisc) for efficient germline editing. If the injected mix is injected into the yolk it may become inefficiently distributed into the blastomere, and germline transmission may occur at a lower frequency. Inject up to the end of one-cell stage (35 min postfertilization (mpf); *see* **Notes 10** and **11**).
5. Remove unfertilized embryos by sorting for regular cleavage pattern during the early cleavage stages. Keep a subset of

uninjected embryos to use as a negative control when screening for somatic mutations.

6. Grow the embryos (injected and control uninjected) at 28.5 °C in E3. During the day check the embryos and remove any dying or dead embryos to prevent spoiling of the embryonic medium, which may cause developmental delays in the embryos.

3.4 Screening Activity of the Guide RNA in the Zebrafish

Somatic INDEL frequency in founder (injected) embryos and germline transmission can be determined by PCR using a 100 bp fragment with the PAM site near the middle (*see* **Note 12**). An overview of all the generations and crosses is represented in Fig. 6a.

1. 24 hours after injection remove all the malformed and dead embryos from the dish. If the injection was toxic, resulting in high embryo lethality, repeat the injections using a lower concentration of Cas9 protein/guide RNA.
2. Extract genomic DNA separately from each of the eight healthy injected embryos and two uninjected embryos. Place each single embryo in a PCR tube and remove a majority of the E3. Add in 100 μL 50 mM NaOH and incubate for 20 min at

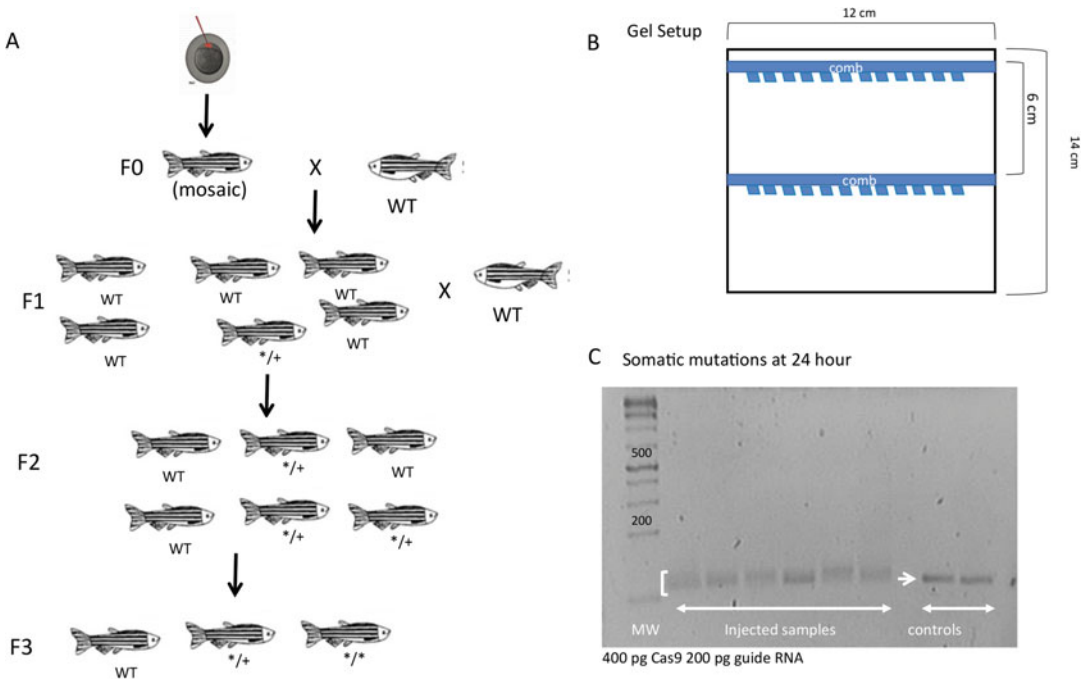


Fig. 6 Screening for somatic and germline mutations in zebrafish. (**a**) Diagram of the fish crosses that are used to isolate a CRISPR-Cas9 mutation in zebrafish. (**b**) Diagram of the gel setup that allows for the screening of somatic mutations 24 h after injection. (**c**) A gel showing somatic mutations in injected CRISPR-Cas9 samples compared to control embryos. The injected embryo band is a smear (bracket) when compared to that of control embryos (arrow). The smear reflects the presence of a mosaic DNA population

95 °C. Cool samples down to 4 °C, add 10 μL of 1 M Tris–HCL pH 8.0, and vortex the samples.

3. Amplify the DNA using primers that create a 100 bp fragment with the CRISPR-Cas9 target site in the center of the fragment.
4. Run the PCR product out on a 2.5% gel at 120 V until the gel resolves or the DNA has reached the end of the lane (6 cm) (Fig. 6b). If the CRISPR-Cas 9 created INDELs a smear will be observed in the injected samples but not in the control samples, as observed in Fig. 6c (*see* **Note 13**).
5. If smears are observed in the injected samples, grow up the sibling injected embryos. If the samples lack a smear, redesign and inject a different sgRNA (*see* **Note 14**).
6. Once the injected F0 fish have become sexually mature, outcross the F0 fish with a wild-type strain of fish to create the F1 generation. The F0 fish will have mosaic germlines, so the alleles will not be recovered in expected Mendelian ratios, and each injected fish has the potential to carry multiple alleles within their germline (Fig. 6a).
7. Extract and run PCR on samples of 15 individually prepped F1 embryos using the 100 bp primers on a 2.5% gel at 120v until the gel resolves (**steps 2–4**). If a germline transmission occurs, double bands (indicating heterozygosity) will be observed. If heterozygosity is observed, raise the remaining of F1 siblings. Outcross the F0 fish a few more times to generate additional backup progeny from which to potentially recover the mutation(s). Raise a minimum of 100 siblings (*see* **Note 15**).
8. Once the F1 fish have reached adulthood, extract DNA from fin clips and individually genotype the fish using the same 100 bp primers (**steps 2–4**, initiating from fin clips), keeping the fish in separate tanks during the PCR. Identify the adult heterozygous fish and outcross them to a wild-type fish. Generate and grow up an F2 generation from identified carriers (*see* **Note 16**).
9. Once the F2 generation has reached adulthood, identify heterozygous fish using the same 100 bp primer pair (**steps 2–4**, initiating from fin clips). The mutation should then be recovered in Mendelian ratios (in the F2 generation: 50% heterozygotes, 50% wild type).
10. Identified F2 heterozygous carriers can be incrossed to generate F3 homozygous mutant embryos, which can be identified by testing individual embryos using the 100 bp PCR analysis on a 2.5% gel until it resolves (**steps 2–4**). Homozygous mutant fish should be recovered in Mendelian ratio (in this F3 generation: 25% mutant, 50% heterozygote, 25% wild type).

11. The DNA from a known homozygous embryo can be used to amplify a larger region flanking the intended mutation site with a different primer pair (about 250 bp), which can be used for sequencing to determine the precise mutation.
12. Once the mutation has been sequenced, examine how it changes the protein using Sequencher or another alignment program. Identify a mutant line (or lines) that has a premature stop codon or a potential frameshift mutation that does not add too many extra amino acids after the frameshift (<10 amino acids).
13. To maintain the line for future generations continue to outcross heterozygous fish to wild-type fish. Outcrosses through generations while maintaining the targeted mutation will gradually segregate away potential off-target mutations in the fish line. In each generation, determine heterozygous carriers through genotyping (**steps 2–4**).

4 Notes

1. If the gene of interest has multiple transcripts make sure that the domain targeted for editing is present in all transcripts.
2. CHOPCHOP will also allow searching for all the guide targets within a gene, but if the guide RNA targets the gene early in the open reading frame there is a possibility that a downstream start codon will allow for the creation of a truncated protein or, alternatively, exon skipping, either of which can result in products that retain partial, dominant negative, or gain-of-function activity. Targeting the first active domain of the protein makes the probability of obtaining a true complete loss-of-function (null) mutation more likely [18].
3. In primers for sgRNA synthesis, mismatches in the first two base pairs of the sgRNA do not appear to have effects on the CRISPR activity [19]. When using a T7 promoter, an additional GG is required at the 5′ end of the sgRNA. If using a SP6 promoter there is no requirement for the 5′ of the sgRNA [16].
4. An SP6 promoter can be used instead of the T7 promoter. If this is the case the gene-specific oligonucleotides will be:

   5' ATTTAGGTGACACTATA-N_{20} GTTTTAGAGCTAGAAATAGCAAG 3'

5. The oligonucleotides can be ordered in standard desalted form with no additional purification.
6. We have had good success using a mini elute PCR cleanup kit (DNA Clean and Concentrator: ZYMO Research) to concentrate the annealed oligonucleotides and have a higher concentration of template for RNA synthesis. Other commercially

available, standard PCR cleanup kits can be used to purify the template too.

7. A successful sgRNA synthesis results in a dominant band of 120 bp RNA after running the sample on a standard 1.5% agarose gel, although bands larger and smaller than 120 bp may be observed. The absence of a band may reflect that the T4 fill-in reaction failed, in which case repeat annealing and T4 fill-in reactions.
8. We use MEGAshortscript kit (Invitrogen) because it is made specifically for transcripts in the 20–500 bp range. A conventional T7/SP6 kit can be used but a lower yield might be observed.
9. Resuspend the newly synthesized RNA in about 20 μL of nuclease-free water. Then dilute it with nuclease-free water to the proper concentration after quantification.
10. A 1 nL bubble can be measured by testing the needle and pressure settings while carrying out a mock injection into a drop of mineral oil laid onto a micrometer slide.
11. Injected Cas9 mRNA, which produces protein after translation in the embryo, can also be used to create INDELs, but in our hands directly injecting the protein results in a higher frequency of lesions [16].
12. There are other methods to screen for activity of the guide, such as T7 endonuclease assay, high-resolution melt assay, and next-generation sequencing [16, 18]. However we find that the PCR method presented in this chapter is simple and highly effective.
13. To resolve the smear in the injected embryos the comb width and gel box size are key. The wider gel comb allows the sample to spread out allowing for increased resolution and observing the smear. The combs we use are approximately 0.625 cm wide and a gel tray is 12 × 14 cm. We have also observed better resolution when detecting the nucleic acid using ethidium bromide than with Sybr Safe. Addition of 5 μL of ethidium bromide per 1 liter of TBE to the positive end of the gel box permits use of the gel for longer amounts of time without signal fading.
14. To control for injection, RNA synthesis, and Cas9 activity, one can inject a published guide RNA (listed below) for the *golden* gene. Successful knockouts in this gene will result in reduced pigment, which will manifest as mosaic patches in the founder generation and through the body in successive, non-mosaic generations. A reduction in pigment will be observed in melanophores throughout the body starting at 30 h after fertilization and through day 5 and later, and temporarily in the eye

retina from 30 to 48 h postfertilization (retinal pigment will recover to wild-type levels). The *golden* guide RNA is as follows:

5'TAATACGACTCACTATA GGTCTCTCGCAGGATGTTGC GTTTTAGAGCTAGAAATAGCAAG 3'

15. More than 15 F1 embryos can be prepped when screening for germline transmission. However, if the mutation is not identified within this sample size, due to significant work in genotyping adults in these cases it is often not worth carrying that line forward and the F1 siblings can be discarded.
16. Once F1 adult heterozygotes have been identified, select 3–6 unique alleles for additional analysis through DNA sequencing. Selecting alleles from different F0 fish allows identification of multiple independent alleles that are more likely to be unique genetic lesions.

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Chapter 24

Modeling Embryonic Cleavage Patterns

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Abstract

The division patterns of early invertebrate and vertebrate embryos are key to the specification of cell fates and embryo body axes. We here describe a generic computational modeling method to quantitatively test mechanisms which specify successive division position and orientation of eggs and early blastomeres in 3D. This approach should serve to motivate and guide future experimental work on the mechanisms controlling early embryo morphogenesis.

Key words Early embryos, Division positioning, Microtubules, Asters, Cell shape, Maternal domain, Yolk

1 Introduction

Early embryo development of both invertebrate and vertebrate species begins with the cleavage period. During cleavage, the egg undergoes a stereotyped choreography of subsequent reductive divisions that transform the one-cell egg into a multicellular embryo [1]. Cleavage patterns are not random, but usually follow a well-ordered set of specific position and orientation of blastomere divisions. Those patterns are most often conserved among large groups of species, such as in amphibians, fishes, and echinoderms, and serve as prime examples of developmental robustness. Mechanisms which control cleavage geometries remain however poorly understood. This is in part because this period is driven by maternal material in the embryo, and thus poorly amenable to genetic screens or manipulations [2]. In addition thorough live imaging of large eggs and blastomeres implicates sophisticated methodologies [3], or may even be impaired given the opacity of many embryos. Factors that have been directly and indirectly implicated in the regulation of division plane positioning during cleavage include cell shapes [4–7], yolk layers [8, 9], and maternal cortical polarity domains [10–13]. Those may affect the organization or dynamic properties of astral microtubules which exert forces and

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_24,

torques to position and orient asters and mitotic spindles that specify division planes.

Recent experimental work in echinoderms, fish, and frog embryos has suggested that division plane specification in large eggs and blastomeres is instructed from interphase/telophase microtubule (MT) asters which fill the large cellular volume, and probe cell shape through length-dependent MT forces [6, 7]. This mechanism aligns the division axis with the cell shape long axis, and has been proposed to serve as a default cue guiding division plane orientation and position in early embryos [14, 15]. Given the presence of asymmetric divisions in some blastomeres of many embryos, cell shape effect may be overridden or biased by additional cues such as vegetal polarity domains in echinoderms or by yolk layers in fish and amphibians, which alter the distribution of MT forces around cells.

We have recently implemented 3D computational models to predict division position/orientation as a function of various inputs [16]. Those models compute in a systematic manner the forces and torques exerted by astral MTs on centrosome pairs at the center of asters and output a mechanical equilibrium which corresponds to the preferred division position and orientation. Those models are highly valuable to dissect the basics of cleavage patterns in many embryos. In here, we report on a set of scripts and methodologies to implement this modeling approach to test the contribution of various cues to the division patterns of early embryos.

2 Materials

Computer equipped with Mathworks Matlab (Version 2015a and above) and Surface Evolver [17]: There is no special requirement for computers, but it is recommended for better performance to have a fast CPU. On a good system a simulation of 10,000 iterations with calculation of a torque map (see here after) typically takes 5–10 min.

Programs may be downloaded from:
Division Prediction Program (DPP):
http://www.minclab.fr/wp-content/uploads/2018/01/DPP-Package.zip

Surface Evolver
http://facstaff.susqu.edu/brakke/evolver/evolver.html

3 Methods

3.1 Generating 3D Cell Shapes

The initial input for the program is the 3D shape of a cell or a group of cells in an embryo, for instance, defined by the 3D coordinates of cell surfaces. This serves as a spatial reference to run a prediction on

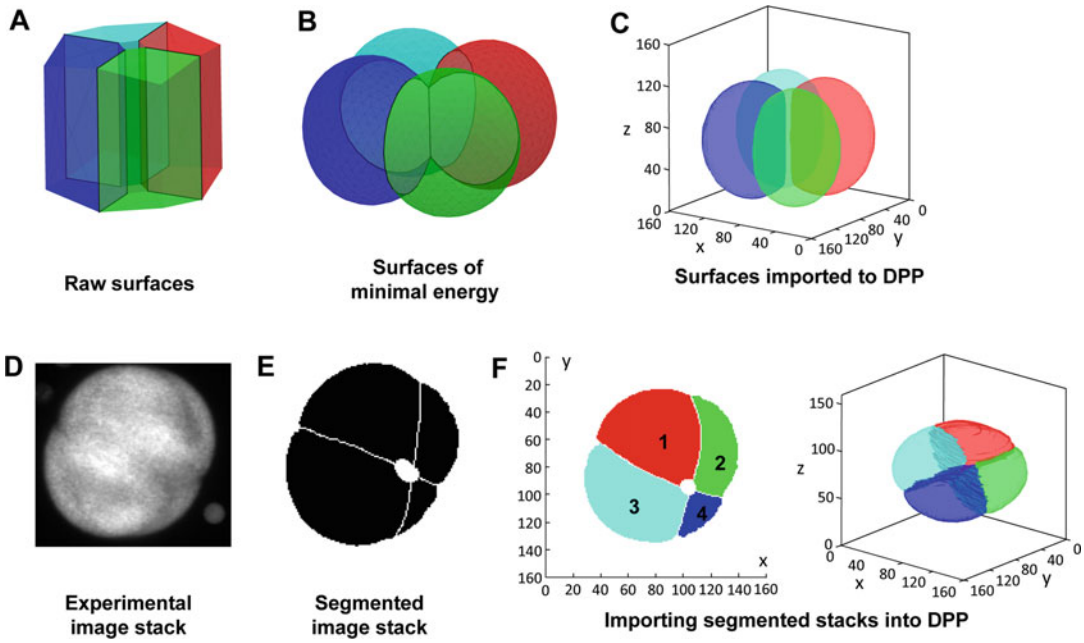

Fig. 1 Creating 3D Cell Shape inputs. (**a**) Raw surfaces for a four-cell embryo in Surface Evolver (**b**)-equilibrated shape with surfaces of minimal energy obtained in SE. (**c**) Equilibrated 3D shape imported to DPP with Matlab. (**d**) Experimental image projected stack of a four-cell sea urchin embryo. (**e**) Segmented image stack. (**f**) Interface to import 3D segmented stack in Matlab for DPP, through manual connection of 2D segments in 3D

the position/orientation of the site of cell division. 3D shapes may be artificially generated using the surface minimization software Surface Evolver [17], or from a 3D segmented stack from labeled embryos imaged with a confocal or a two-photon microscope (Fig. 1).

3.1.1 Generating an Input 3D Shape with Surface Evolver

We here provide some initial guidelines to generate representative 3D shapes that closely resemble real physical egg and blastomere shapes with the software Surface Evolver (SE). This software iteratively finds the surface of minimal energy under given constraints (Fig. 1a, b). Constraints include final volume, surface tension values, and confinement. The language of SE is rich and for details on structures, constraints, macros, or function definitions we refer the readers to its web page.

SE requires input files that contain information on the topology and constraints of the studied surfaces. The topology is described by a simple mesh (Fig. 1a), elements of which are named and organized according to the rules of SE. The mesh describing topology is defined by vertices and their coordinates, oriented edges linking two vertices, oriented faces consisting of three edges, and bodies consisting of N faces. The constraints describe the behavior of the surface; for example coordinates can

be restricted to an arbitrary space (like a cylinder) or additional energy may be assigned to arbitrary faces, to mimic effects of differential surface tension around adhering blastomeres for instance [16]. We provide several input files in the folder DPP package\DPP files\generating_shapes_from_SE\generic shapes\, which can be used to create one-cell spheres, rods, and typical two-cell, four-cell, or eight-cell stage embryos. In the DPP package, we further provide more detailed guidelines to create other shapes and constraints with SE. For the method description below we will use the example file "4 cells - confined in sphere - raw.fe".

Creating Shapes with Surface Evolver

1. Open Matlab. Navigate to the DPP Package folder in Matlab, right-click on it, and click "add to path/ Select folders and subfolders". Navigate to the folder "DPP package\DPP program\" where three scripts named "launcher" are placed; you will work from here.
2. Open the script "launcher_1_generate_shapes.m" with Matlab. Set the path where you will store the shapes, simulation parameters, and results. For instance, if you want to store your cells and their simulations in a folder "C:\Data\my cell" you set the variable root_path = "C:\Data\my cell".
3. Run the function that generates the folders shape_paths = f1_generate_paths(root_path) by selecting the two abovementioned lines and pressing F9 to evaluate/run. This will create the folder "C:\Data\my cell" and inside it a folder "C:\Data\my cell\shape_source". In the latter you will keep all cell shape-related files.
4. Copy the file "4 cells - confined in sphere - raw.fe" into the folder "C:\Data\my cell\shape_source" and double-click the input file to open it in SE.
5. To see the surface, press "s" to enter the graphic mode (Fig. 1a). Then "q" to quit the graphic mode and return to SE command terminal. You may zoom in and out (press Z when graphic window is active) or rotate (press R when graphic window is active) the surface using the mouse.
6. To iteratively find minimal energy configuration (Fig. 1b), the user needs to perform subsequent refinements of the mesh and calculation of novel surface coordinates. Type "r" to refine the initial mesh (subdivide); this will increase the number of vertices. Then "g10" to make ten iterations of surface minimization (g100 will make 100, etc.). Refine the mesh again with "r". Refine and reiterate calculations several times until you find that the shape is stable and does not change significantly with the following iterations. As a start for division prediction, we recommend to refine the mesh to reach a number of typically 1000–2000 vertices.

7. Save the final file by typing "d" to save the data in one file in SE format. Use a different name than the original file, e.g., "4_cell_final".
8. Type "export_lists()" in the SE terminal to extract the data in separate lists for vertices, edges, faces, and bodies. Note that this function has been added to the example files to facilitate the following steps, but is not a default function of SE. In the package we provide this function which can be copied into any SE function. At the end of this process, you should end up with two SE files (.fe), one for the initial shape, and one for the final, and several .txt files for edges, faces, vertices, and bodies.

Translating the SE Surface into a Matlab Matrix with Labeled Bodies

1. In Matlab open the script "launcher_1_generate_shapes.m". Find the function "f2_extract_bodies_from_SE(shape_paths, [X, Y])" and run it by selecting the line and pressing F9. The numbers in the brackets are the size in pixels of a Matlab matrix that will comprise the SE surface; for instance: [X, Y] = [200, 240] pixels. Any size in any proportion can be used, but large matrices may take much longer to simulate. Typically, we use matrices of the sizes ranging from 150 × 150 to 200 × 200 pixels; X and Y may be set unequal (Z is automatically recalculated from shape's dimensions and given X and Y to ensure the best fit of the shape within the matrix).
2. Matlab will ask you to provide the name of the SE file from which you exported the lists (its name was used as a part of those lists and will be used to find them); in our case it is "C:\Data\my cell\shape_source\4 cells - confined in sphere - raw.fe". The program will read the raw coordinates in ".txt" files and save them as "C:\Data\my cell\shape_source\shape_data.mat", after which it will translate them to matrix coordinates, label them according to the body lists, and save those as "C:\Data\my cell\shape_source\labeled_bodies.mat" in the same folder.
3. In Matlab open the script "launcher_1_generate_shapes.m". Find the function "f3_show_labeled_bodies(shape_paths)" and run it. It will find the file "labeled_bodies.mat" and show the labeled bodies (Fig. 1c). If everything went fine up to this point, the system is ready to run division predictions (Subheading 3.2).

3.1.2 Generating an Input 3D Shape from an Imaging Stack of a Real Embryo

In many instances, one may need to input the direct 3D shape from a cell or a group of cells in an embryo/tissue, and compare experimental division orientation with model predictions. Those shapes may be experimentally obtained from a Z-stack taken on a labeled embryo with confocal, light-sheet, or two-photon microscopy (Fig. 1d). Here we solely provide a method to convert a 3D

segmented stack into an input file to run the division prediction program. Segmentation of 3D shapes may be achieved with ImageJ or Imaris, and will largely differ depending on the embryo, labels, and microscopes. For our purpose, input segmented stacks must be black inside and white outside. The voxel should be cubic (it should span equally in all dimensions: $V_x = V_y = V_z$) and the final segmentation is recommended to span not less than $100 \times 100 \times 100$ pixels (Fig. 1e).

In the folder "DPP package\DPP files\generating_shapes_from_experiment\" we provide an example stack of a segmented four-cell sea urchin embryo called "4CellUrchin_final.tif" which we use to present this part of the method.

1. Open Matlab. Navigate to the DPP Package folder in Matlab, right-click on it, and click "add to path/ Select folders and subfolders". Navigate to the folder "DPP package\DPP program\" where three scripts named "launcher" are placed; you will work from here.
2. Open the script "launcher_1_generate_shapes.m" with Matlab. Set the path where you will store the shapes, generated cells, and their simulations. For instance, if you want to store your cells and their simulations in a folder "C:\Data\my cell" you set the variable: root_path = "C:\Data\my cell". Run the function that generates the folders shape_paths = f1_generate_paths(root_path) by selecting the two abovementioned lines and pressing F9 to evaluate/run. This will create the folder "C:\Data\my cell" and inside it a folder "C:\Data\my cell\shape_source". Copy the file "4CellUrchin_final.tif" into this folder.
3. In Matlab open the script "launcher_1_generate_shapes.m", find the function "f2_extract_bodies_from_IJ", and run it by selecting the line and pressing F9. It will offer to choose a segmentation stack; choose "4CellUrchin_final.tif".
4. You will be taken to an interface allowing you to manually connect 2D regions in the stack to reconstitute the proper 3D shape of your embryo/tissue (Fig. 1f). For this, you will need to manually label 2D regions belonging to one cell from slice to slice (Fig. 1f, left). You can scroll through the whole stack using the mouse wheel. Then set a label number N, by pressing a numeric key on the keyboard, and label a 2D region with this N number by clicking on each z-slice as it appears. The program takes the user to the next slice automatically after the click. After all slices of one cell have been connected, you can visualize the result in 3D by pressing <v > (Fig. 1f, right). After all cells have been reconstructed, it is recommended to clean the stack from lone pixels by pressing <c > and visualize in 3D again; the final result should look similar to Fig. 1f. Export the resulting bodies by pressing <s>. This will create

a Matlab file "C:\Data\my cell\shape_source\labeled_bodies.mat". If everything went fine up to this point, the system is ready to run division predictions (Subheading 3.2).

3.2 Predicting Division Position and Orientation in 3D

In this part we describe the methods to use the scripts which allow to make a prediction on the orientation and position of the division axis. This prediction will have as inputs 3D cell shapes defined as in the preceding paragraphs, and parameters, which relate to the distribution of astral microtubule (MT) forces within cells and to simulation procedures. As such, it is important to state that those models can solely be useful to study cell division in cell types and systems in which MTs are prime contributor of nuclei/spindle position, as in most vertebrate and invertebrate early embryos [15, 14]. In our model, MT forces may depend on the spatial distribution of MTs around centrosomes, the length of MTs, or the presence of cortical domains. MT lengths are defined by cell shape and/or presence of MT excluding structures in cells, such as neighboring asters or yolk.

3.2.1 Setting a Choice of Parameters for the Simulations

1. In Matlab open the script "launcher_2_generate_cell.m", which contains a set of parameters for the cell simulation. If you run this program following immediately Subheadings "Creating Shapes with Surface Evolver" or "Translating the SE Surface into a Matlab Matrix with Labeled Bodies", the path to your cell is already set. Else, redo Subheading "Creating Shapes with Surface Evolver", **step 2** or Subheading "Translating the SE Surface into a Matlab Matrix with Labeled Bodies".
2. Define cells to simulate: "body_ind =". If you have several cells (like in the four-cell embryo mentioned above), this is the index of the particular cell that will be used as the shape input. In the part described in Subheading 3.1.2, **step 4**, to generate shapes from real 3D images, this number is the one defined by the user during the 3D reconstruction.
3. Define microtubule pulling force parameters. As described in [16], the general formula used to define the force exerted by each MT in the asters is

$$F = L\text{^Beta1} + \text{pullfactor}^{*} L\text{^Beta2} + \text{pullfactor\_exp}^{*} 2(L/\text{Unit\_branch})$$

The first term corresponds to pulling in the cytoplasm in proportion to MT length, with an exponent factor, "Beta1", reflecting putative non-linearities, which by default is set to 3 [6]. The second and third terms reflect pulling from a polar cortical domain (whose location and size are defined hereafter). "Beta2" reflects an exponent for cortical pulling, and "pullfactor" the strength of this effect. Beta2 is set by default to 2 to reflect a situation in which diluted motors are limiting pulling

from the domain [18]. The last term reflects a situation in which MT number at the domain is limiting pulling, and possesses an exponential dependence term, defined through the parameter "Unit_branch", to mimic the presence of branching in large embryonic asters (a situation likely irrelevant for somatic cells). This parameter has a default value of 6.75, as experimentally measured in sea urchins [6]. The strength of this term is defined by the parameter "pullfactor_exp". If both parameters "pullfactor" and "pullfactor_exp" are set to 0, then the script will not propose you to position and size a cortical domain. In such case, you may directly proceed to Subheading 3.2.1, **step 7**.

4. Define exclusion regions for microtubule growth. Microtubule effective length can be corrected for the presence of intracellular structure such as yolk which affects MT growth in a dose-dependent manner. This is done by correcting the angular density of MTs in asters by a factor 1-[yolk]/η, with [yolk] the local yolk concentration and η a sensitivity parameter for how MTs are affected by the yolk. This implies defining the location of the yolk gradient, its orientation, and its sharpness, as detailed in [16]. The parameter "gradyolk" defines the sharpness of the yolk gradient (if set to 0, MTs are uniform inside the cell shape). "erfoffset" is the offset of the sharpest gradient point in pixels. "direction_grad" is used for the direction of the yolk gradient, and is defined by polar (θ) and azimuthal (φ) angles, in degrees, counted in a standard convention: θ_y is counted from the z-axis; φ_y is counted counterclockwise from the x-axis (Fig. 2b). "MTstab" defines the sensitivity of MTs to yolk, η, and is set to 1 by default. Importantly, the shape of the yolk gradient will appear in the graphic interface upon running the script, and can thus be adjusted back (Fig. 2d and Subheading 3.2.1, **step 8**).

5. Aster parameters: Parameters related to MT asters include initial position and orientation of asters from where to start a simulation, extension angle of asters, and distance between centrosomes. "anglelimit_deg" reflects the extension of asters in degrees (β in Fig. 2a). "theta" is the starting polar angle in spherical coordinates, counted from the z-axis (θ in Fig. 2a). "phi" is the starting azimuthal angle in spherical coordinates; counted counterclockwise from the x-axis (φ in Fig. 2a). "startingxyz" is the initial position of nuclei/spindle center in pixels, defined by three Cartesian coordinates [x, y, z]. If empty, the initial position will be at the calculated "centroid" of the cell shape. "Nucsize" is the distance between centrosomes in pixels (spindle or nuclear size). "Nucexcl" is a parameter used to calculate an exclusion volume ensuring that the nucleus/spindle stays inside the cell during the simulation. Nucexcl cannot be <1; typical values are 1.2–1.4.

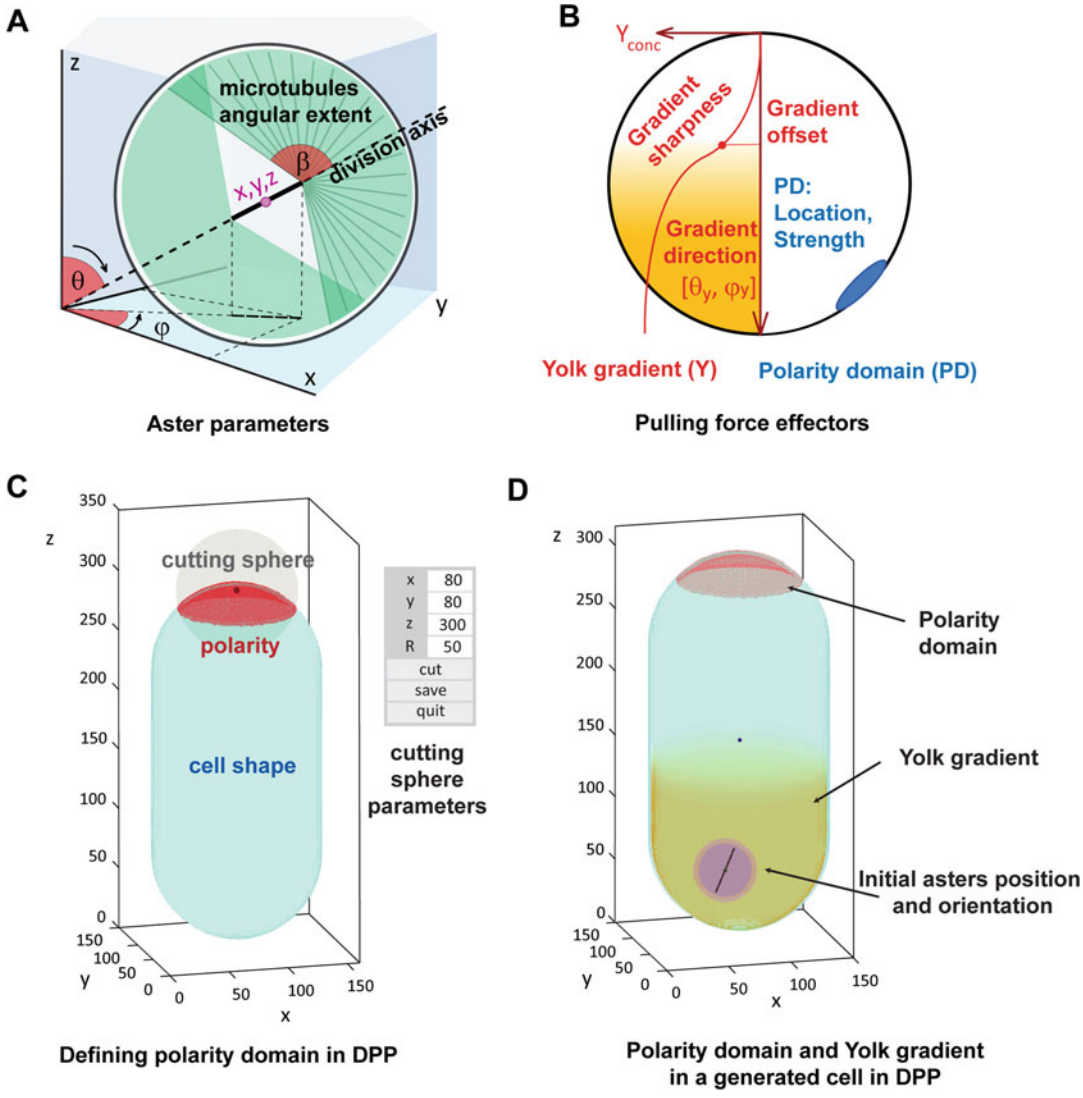

Fig. 2 Input parameters for predicting division position and orientation. (**a**) Microtubule aster parameters. Polar and azimuthal angles (θ and φ) defining the division axis angle; and position of the center o (x, y, z); angular extent of microtubules (β, counted from the division axis). (**b**) Parameters affecting microtubule pulling forces. MT growth may be tuned by the presence of yolk gradients (Y), with the concentration gradient defined by a direction (θ_y, φ_y), an offset from the shape centroid (along the gradient direction), and sharpness. A polarity domain (PD) with a given size, location, and MT pulling strength may also be added as an input for simulations. (**c**) Matlab interface to create a polarity domain: a cutting sphere of defined center and radius intersects the 3D cell shape to define the domain. (**d**) Visualization of the cell with its major simulation parameters used as initial inputs to predict division position and orientation

6. After all the parameters have been set, run the whole script by pressing the "Run" button in the Matlab Editor panel. This will create the parameter structures and feed them to the class "DPP_cell" needed for the simulation.
7. If you have set polarity parameters different than 0 (*see* Subheading 3.2.1, **step 3**), you will be offered to position and size a polarity domain. For this you will be brought directly to a graphic interface (Fig. 2c). The domain is defined as the intersection of the 3D cell shape with a cutting sphere. Use the interface to define values for the coordinates (x, y, z) of the sphere center and radius (R), and press "Cut" to visualize the domain. Once set, press the button "Save" to set the domain for the simulation. If you press "Quit" this will set the pulling pre-factors to 0 and run a simulation purely based on cytoplasmic MT forces and cell shape.
8. If everything was set correctly, the cell with a polar domain, a yolk gradient, and an initial orientation/position of nuclei/spindle will be shown as in Fig. 2d. Corresponding parameters will be automatically saved in a new folder "cell_n". Each time you run this script the cell folder index "n" increases automatically, e.g., "C:\Data\my cell\cell_1" and "C:\Data\my cell \cell_2".

3.2.2 Starting a Simulation

The principle of the simulation is based on a random walk around the 3D position and orientation of the nucleus/spindle by three center coordinates (x, y, z) and two angles (θ and φ). At each iteration one of those variables is changed, the force and torques of all MTs on the nucleus/spindle are calculated and compared to the previous step in order to converge toward an equilibrium of minimal forces and torques [16]. The simulation thus comes with parameters to optimize time, precision, or accuracy, which need to be defined by the user.

1. Following the preceding section, open the script "launcher_3_-generate_simulation.m".
2. Set visualization parameters.

   With "show_log = 1" the program outputs to the Matlab command line information on what parameter has been varied and how forces are calculated. If not required, set to 0.
   With "show_visually = N" (higher than 0) the program will display the nucleus position and orientation of each N iterations. If set to 0, there will be no visualization, which may improve simulation speed.
3. Autosave flags. Set to 1 if needed and 0 if not required.

   "auto_save_nucleus_run = 1": the program will save the run of aster centers (trajectory during simulation) as a PNG picture.

"auto_save_torque_map = 1": the program will save a torque map as a PNG (*see* below). "auto_save_state_history = 1": the program will save the history of varying parameters during the simulation as a PNG.
"auto_save_split_image = 1": the program will save an image of a divided cell (with two daughter cells) as a PNG.

4. Random walk parameters.

   "angle_btw_mt_deg" defines the angle (in degrees) between microtubules (used for both MT polar and azimuthal angle), and somehow reflects the precision of the simulation. If set to 10, for instance, the forces will be calculated along MTs in the aster spanning each 10° in both polar and azimuthal angles. The larger the angle between MTs, the faster the calculations are, but the less precise also. It is recommended to use large steps (10–20) to quickly and roughly find equilibrium position and then use lower angles (1–3) to refine this position and find proper orientation (*see* below).
   "jump_chance =": Probability of jumping to a new configuration, even if it has been estimated to be less favorable (forces and torques are higher) than the previous state. If set to 10, then the chance to make a jump is 10%.
   "Inertia =": It is the probability to vary the same parameter in the same manner if its previous variation led to a more favorable state (100: always use previous variation; 0: randomly pick a new variation).
   "duration_equilibrium =": The number of iterations required for a new state to be tested for equilibrium. If x, y, and z do not change for this number of iterations, a position is considered equilibrated.
   "duration_full =": It sets the maximum number of iterations (use typically >5000).

5. The torque map is important as it informs on the stability of the current division orientation as compared to others. For instance in shapes which are roughly isotropic, this map would be flat with no sharp torque minima [16]. The torque map is a 2D matrix of absolute values of torques at each orientation angle (Fig. 3d), calculated as:

$$T(\varphi_i, \theta_j) = \sqrt{T_{\varphi_i}^2 + T_{\theta_j}^2}$$

   Torque map parameters are as follows:
   "torque_map_angle_step =": This sets a precision step for torque map calculation. If set to 10, torque map calculation will sample division orientations each 10° for both θ and φ.
   "auto_calc_torque_map = 1": The program will calculate a torque map automatically after the simulation (it may take a long time depending on the precision of the map). If not required, set to 0.

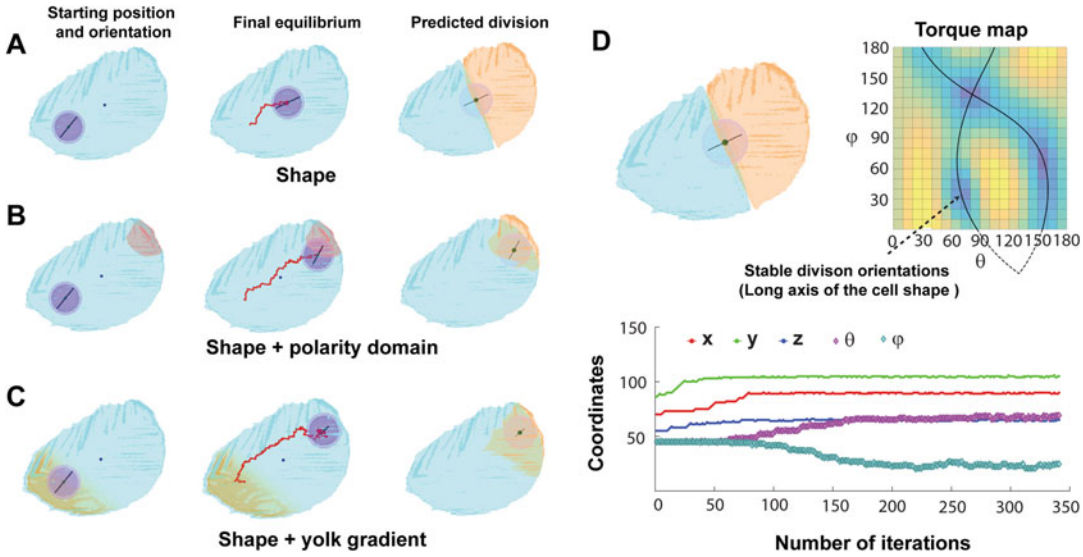

Fig. 3 Example of DPP simulation results. (**a**) (Left) Cell shape corresponding to one blastomere of the four-cell embryo from Fig. 1d is used as the sole input to predict division position and orientation. (Middle) After simulation, asters are positioned at the cell center and oriented along the long axis of the 3D shape. (Right) The predicted divided cell is then generated. (**b**) Same as in (**a**) but with the presence of a polarity domain pulling on asters. Note the strong asymmetry in cell division. (**c**) Same as in (**a**) but with a yolk gradient which creates a shift in the final position of asters away from yolk layers. (**d**) Simulation as in (**a**) with corresponding torque map, computing the torque exerted on asters as a function of all possible 3D angles. Torque minima correspond to stable orientations, highlighted by the black lines. (Bottom) Evolution of angular and position coordinates during the random walk, as a function of iterative step number of the walk

6. Once parameters are set, start the simulation by selecting all the lines from "current_simulation" down to "current_simulation.run" and pressing F9.
7. The simulation will end either if it ran out of iterations (from the maximum number allowed) or if it found an equilibrium which did not move during a number of iteration set by the parameter inertia. The final state of the simulation will be saved automatically in the cell folder, i.e., "C:\Data\my cell\cell_n\" with the prefix corresponding to the index of current instance: "run_1", "run_2", and so on. Windows showing the results of the simulation will open, depending on the autosave flags (*see* Fig. 3 for examples of simulation output).
8. If you want to refine the simulation, with more MTs to improve precision, or with a more detailed torque map, use the section "Refine division orientation", insert new numbers, and run by selecting lines from "current_simulation.reset" down to "current_simulation.show_division_plane".

9. Finally, one can use old cell shape and parameters to rerun simulation. To that aim, open the folder with the cell of interest, e.g., "C:\Data\my Cell\cell_1", and drag and drop the file "cell.mat" into Matlab workspace to load it. In the script "launcher_2_generate_cell.m" run the line "current_cell.show", and visualize the initial state of the cell visually. You can also inspect all the properties of the cell by typing "current_cell" in the command line and pressing enter. Then restart from Subheading 3.2.2, **step 1** to run a new simulation.

Acknowledgments

We thank Anaëlle Pierre and Benjamin Blanchard for their initial contribution to the model. This research was supported by the CNRS and grants from the "Mairie de Paris emergence" program, the FRM "amorçage" grant AJE20130426890, and the European Research Council (CoG Forcaster N° 647073).

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Chapter 25

Laboratory Guidelines for Animal Care

Marcelo Couto and Charles Cates

Abstract

Animal research is a controversial subject because of the ethical and moral implications of using unwilling research subjects in potentially painful or distressful procedures usually ending in euthanasia. As such, it must be conducted in a compassionate and responsible manner geared toward maximizing the animals' quality of life prior to and during experimentation. Because of its contentious nature, the conduct of animal research is highly regulated at the federal, state, city, and institutional levels. It is essential that researchers acquire a thorough knowledge of the procedures to be conducted as well as a working knowledge of the regulations. This will maximize humane care of research animals and prevent potentially negative or detrimental interactions with groups opposed to using animals in biomedical research. Perhaps the best way to attain these goals is to avoid inadvertent instances of noncompliance with their research protocol or applicable regulations. Regulatory noncompliance can also have serious negative consequences on investigators' research careers ranging from temporary suspension of their protocols to loss of funding and revocation of principal investigator status and associated privileges. To minimize such adverse outcomes, it is advised that researchers build positive and collaborative relationships with key institutional players such as the veterinary staff, the Institutional Animal Care and Use Committee (IACUC), and top administrators. Guidance is provided regarding the appropriate handling of regulatory noncompliance.

Key words Animal research, Ethics, Animal facilities, Organizational relationships, Animal welfare regulations, Regulatory compliance

1 Introduction

This chapter is intended to provide useful background information and practical advice to investigators using animals in research, teaching, training, and testing, herein described as "research." The topics covered will include an overview of the ethics of the use of animals in research, organizational infrastructure, and key stakeholders in a research animal care and use program, applicable regulations, different types of animal facilities, and proper handling of regulatory noncompliance. It should be noted that occupational health and safety risks are inherent in the course of working with animals in a research setting. Nevertheless, a thorough discussion of hazard identification and risk assessment is beyond the scope of this

Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2_25,

chapter; investigators are directed to consult with their institutional occupational health and environmental health and safety representatives for specific institutional requirements.

The use of animals in research is a privilege and must be conducted responsibly. As opposed to human clinical trials, where patients volunteer for a clinical study and sign an informed consent, laboratory animals obviously do not voluntarily participate in research activities and therefore require the intervention of advocates, such as the researchers, the veterinary staff, and the Institutional Animal Care and Use Committee (IACUC) [1]. Positive and collegial interactions and collaboration among researchers, the veterinary staff, the IACUC, and the institutional administration are essential for the safe, effective, and responsible conduct of animal research.

It is vital that the well-being of the animals be ensured to the maximum extent possible during the conduct of research. This requires balancing research goals with the compassionate use of the animals. By and large, current animal research is conducted responsibly. This was not always so, however. Before and during the 1960s, a relatively small number of experimenters were involved in mistreatment of animals as well as questionable practices for acquiring their animal research subjects. When this information became public, people were justifiably enraged. Stories of animal abuse and stolen pets flooded the evening news and the press [2]. In response to public outrage and mounting pressure from animal advocacy groups, Congress passed in 1966 the Federal Animal Welfare Act [3]. This legislation effectively sanctioned the humane care and use of animals and established tight oversight mechanisms.

Although a detailed knowledge of animal research regulations and guidelines is not strictly required, all scientists and staff working with research animals would do well to become acquainted with, and have a working knowledge of, applicable animal welfare regulations as well as institutional policies and procedures. In addition, it is highly recommended that primary responsible research personnel (e.g., principal investigator [PI] and lab associates) contact the institutional attending veterinarian in the early planning stages of their research not only to learn about the logistics of the particular animal facilities (e.g., availability of housing and procedural space, security access requirements, hours of operation, lighting schedules, and other possible restrictions) but also to seek assistance and advice with experimental design of studies, writing of grants and animal protocols, and getting the lab ready for mandatory inspections and site visits by the *IACUC* and the *US Department of Agriculture (USDA)* and the *Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International* (*see* below) [4]. Taking such initiatives, in addition to familiarizing yourself with the topics in this chapter, will contribute

greatly to your success in navigating the complex environment of an animal care and use program. We begin our review with a look at the underlying driving force behind regulatory oversight: the ethics of the use of animals in biomedical research.

2 Ethics of Animal Use in Research: Awareness and Appreciation

From a combined experience of several decades in the laboratory animal field, the authors find that the humane and responsible use of animals in research is not only justified but also vital to advancing discoveries leading to improvements in both human and animal health. Numerous examples of the important contributions of animal research abound. Such include the discovery of insulin in dogs, tuberculosis treatments in guinea pigs, cardiovascular advances in swine, diagnostic and therapeutic antibody development in rabbits and mice, and infectious disease and cancer treatment advances in rodents. Many of these developments originally intended to improve the human condition have also translated to major advances in veterinary care of animals, from our precious companion dogs, cats, and other pets to improved condition of horses, livestock, and poultry.

It is assumed that most readers of this text have at some point in the past considered the use of animals in research and come to the decision that (in at least some circumstances) research using animals is warranted. The ethics of animal use in research has been studied and debated in depth for many years and many frameworks have been described for approaching this controversial issue [5–8]. The reader is highly encouraged to investigate literature in this regard, however, as this section is not intended to provide an in-depth analysis. Rather, it serves to bring awareness and appreciation to the fact that the controversy surrounding the use of animals in research drives much of the daily effort the researcher will face in dealing with regulatory burden when conducting animal research. Such regulatory oversight (which we cover in depth in the ensuing sections) represents society's effort to balance such ethical points of view and ensure that living creatures are treated with thoughtfulness and care while justifying use of animals in research with the hope and intent of contributing to a greater good.

In the United States, opinions about the use of animals in research are generally divided 50% *for* and 50% *against* with somewhat more support from males and persons with increased levels of scientific education and less support from females and persons with less scientific background [9]. Nevertheless, the United States currently has a rich and robust biomedical research enterprise. Current estimates suggest that project funding reaches over US $158 billion per year when combining public and private investment with many of these activities involving the use of animals

[10]. The exact number of animals used in research in the United States is challenging to know; however, some estimates put the number as high as 26 million animals per year when including all species [11]. This of course demands that researchers, caregivers, legislators, and regulators alike function as dedicated stewards of these valuable resources. The moral and concerned scientist should take note and make every effort to communicate the importance of animal research to their staff and emphasize the criticality of performing animal research in an ethical and caring manner—not only to withstand scrutiny from activists and regulators, but also to obtain accurate scientific results from responsible studies unmarred by physiological aberrations from avoidable pain and distress. With these considerations in mind, we move on to address practical elements of implementation and execution of a successful and humane animal care and use program.

3 Primer on Regulations, Standards, and Guidelines Regarding Laboratory Animals

Animal research is tightly regulated via numerous laws, regulations, policies, guidelines, and agencies [3, 4, 12, 13]. Rather than dictating how research should be conducted, the intent of the regulations is twofold: (1) minimize the use of animals in research and (2) enhance the quality of life of research animals.

3.1 The "3Rs"

The spirit of the regulations is embodied by the principles of animal experimentation known as the "3Rs": replacement, reduction, and refinement [14].

Replacement refers to the use of nonanimal alternatives in research, such as in vitro methods and mathematical or computer (in silico) modeling. Its interpretation may be extended to the replacement of higher animal species with phylogenetically lower species. The latter is based on the assumption that animals with less developed central nervous systems are less likely to feel pain, distress, and other negative sensations.

Reduction means that the number of animals used in research should be reduced to the lowest necessary that will yield scientifically and statistically significant data. This principle applies to both minimizing animal numbers in individual studies and avoiding *unnecessary* duplication of published results.

Refinement refers to the efforts toward minimizing animal pain, distress, discomfort, fear, boredom, and other negative experiences during the conduct of research. Refinement is achieved through the provision of proper animal husbandry, environmental enrichment, handling, restraint, and appropriate use of drugs, such as anesthetics, analgesics, and tranquillizers. Proper training of investigators is paramount in maximizing refinement.

3.2 Key Points Regarding Regulations

Regulations and standards can be confusing, as their applicability varies with the species of animal under consideration, the source of funding, and the institutional accreditation status. Although there is considerable regulatory overlap, the vast majority of responsible institutions follow the stricter standards. The regulatory maze of animal research is easier to navigate if we keep in mind a few key points:

3.2.1 Some "Rules" Have the Force of Law

The *Animal Welfare Act* (AWA) and associated *Animal Welfare Regulations* (AWR) [3] are administered and enforced by the USDA. The *Health Research Extension Act of 1985* [15] and associated *Public Health Service (PHS) Policy* [12] and *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* [16] are administered and enforced by the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health (NIH). In addition, some states require the filing of special permits. For example, California's Department of Fish and Wildlife mandates that *Restricted or Detrimental Species* permits be filed for the housing or breeding of *Xenopus* frogs, transgenic zebrafish, and other exotic or genetically modified species [17].

3.2.2 Some Non-legislated Standards Have Become de Facto Law

The Institute of Laboratory Animal Research (ILAR), a branch of the National Academy of Sciences, publishes the *Guide for the Care and Use of Laboratory Animals* [13] (usually referred to as *The Guide*) with support from the NIH, the USDA, and the Department of Veterans Affairs. AAALAC International, a private, nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment of animal programs, uses *The Guide* and other animal research regulations as its reference documents and publishes useful position statements on best practices with regard to animal care, staff training, and occupational health [18].

3.2.3 Regulatory Applicability

Whereas the PHS Policy and *The Guide* apply to all live vertebrate animal species, the AWA and AWR apply only to what is known as "USDA-covered species." The AWR defines "animals" as *"any live or dead dog, cat, nonhuman primate, guinea pig, hamster, rabbit, or any other warm-blooded animal, which is being used, or is intended for use, for research, testing, experimentation, or exhibition purposes, or as a pet."* The AWR explicitly excludes *"birds, rats of the genus* Rattus, *and mice of the genus* Mus, *bred for use in research, horses not used for research purposes, and other farm animals, such as, but not limited to livestock or poultry, used or intended for use as food or fiber, or livestock or poultry used or intended for use for improving animal nutrition, breeding, management, or production efficiency, or for improving the quality of food or fiber."* It is worth noting that "*rats of the genus* Rattus, *and mice of the genus* Mus, *bred for use in*

research" comprise the vast majority of laboratory animal species currently used in the United States. The AWA and AWR are silent on considerations for embryos and fetuses, however. NIH/OLAW has only addressed this in the context of hatching animals: animals are covered by PHS Policy only after they have hatched; however, standard procedures are expected to be in place in the event of unexpected hatching [19, 20]. In light of this room for interpretation, it is recommended that any proposed activity involving non-human animal embryo or fetuses be brought to the attention of institutional officials (typically the IACUC if one is in place) and the veterinarian associated with the research program. Furthermore, the use of human embryonic stem cells in animals is a different consideration altogether and is discussed briefly in Subheading 6.1.

3.2.4 Institutional Self-Regulation

Both the AWR and the PHS Policy mandate that the highest organizational authority (i.e., the CEO, President, or Chancellor) be designated as the Institutional Official (IO) ultimately accountable for the animal program. Since top officials are typically far removed from the animal research enterprise, they frequently delegate the IO authority to a Vice President or Vice Chancellor. The IO is charged with appointing an in-house animal ethics committee, commonly known as the Institutional Animal Care and Use Committee (IACUC). The IACUC comprises a diverse group of scientists, veterinarian(s), and laypersons who are charged with ensuring humane care of animals and adherence to regulations governing animal care and use. Most typically, scientists are exposed to IACUCs by way of seeking approval for proposed animal use protocols as well as semiannual inspections of animal procedure or housing areas in laboratory space. It is important to note that the IO may not interfere with or overrule decisions made by the IACUC.

Both the IACUC and the attending veterinarian are directly responsible to the IO and are collectively charged to manage the research animal care and use program. In USDA's interpretation of the Animal Welfare Act, the "*Institutional official* (is) *the individual at a research facility who is authorized to legally commit on behalf of the research facility that the requirements of* (the Animal Welfare Regulations) *will be met*" [3]. In addition, the Public Health Service Policy defines the Institutional Official as *"An individual who signs, and has the authority to sign, the institution's Assurance, making a commitment on behalf of the institution that the requirements of this Policy will be met*" [12]. Nevertheless, perhaps the most useful and descriptive statement asserts that "(t)*he IO must have administrative and operational authority to commit institutional resources to ensure compliance with the PHS Policy and other requirements*" [1]. This interpretation succinctly summarizes that

ultimate authority and responsibility for ensuring humane care of research animals rest with the IO.

3.2.5 A Cautionary Note

Despite the regulatory mandates and assurances, at times the IO lacks sufficient authority, financial discretion, or institutional influence to ensure the provision of adequate resources to the IACUC and the animal care program. This can be an exceedingly frustrating experience for all stakeholders, including researchers. Under-supported IACUCs and animal care programs are unable to secure sufficient, qualified staff and provide them with the necessary training and tools to discharge their duties properly. Under-supported animal facilities often operate with aging and deficient or deteriorating infrastructure and equipment. As such, they fail to provide for an acceptable housing environment for animals, mainly in terms of temperature control and adequate ventilation. Adverse environmental conditions can impact both the welfare of animals and personnel and compromise the integrity of research studies. Failure of major equipment, such as cage washers, sterilizers, laminar flow cabinets, and ventilated cage racks, can further compromise the quality of the research operation by increasing the vulnerability of the animal colony to contamination.

On a strictly financial note, animal research enterprises rarely achieve full cost recovery, especially in smaller, not-for-profit, organizations. Even under the best of circumstances, only 60–70% of the facility's operating costs are recovered as income from per diem charges to investigators [21]. More often than not, in order to break even, animal resource departments require core financial support from the institution in the form of supplementary funds. This support is, of course, in addition to the facility's fair allocation of indirect cost recovery funds. Inability of the IO to secure these additional funds inevitably leads to higher per diem rates for investigators. While some institutions may be more adequately supported than others, it pays to be aware of these fiscal challenges which are likely to be encountered at some point in one's career.

4 Establishing and Managing Key Relationships

As indicated previously, the importance of establishing a positive, cordial, and professional relationship with the veterinary service, the IACUC, and the administration cannot be overemphasized. Take time to get to know them in person during the planning of studies and ask for input with grant and protocol writing. Waiting until a problem arises may start the relationship on a negative note and set the wrong tone going forward. The benefits derived from developing trust and rapport will more than pay off, likely resulting in greater cooperation and assistance with experimental design, protocol reviews, and grant transfers. No doubt, this will save

time, money, and aggravation down the road as well as minimize research delays and noncompliance incidents. It is also important to spend some time learning about services offered by the veterinarian or the animal technical staff (e.g., breeding, injections, drug administration, surgery, pathology, laboratory tests). Knowing that experimental animals are in the competent hands of husbandry and veterinary staff members will give researchers peace of mind and allow them to focus their time and energy on their specific research.

The veterinary staff will welcome your help with identifying and reporting animal health or husbandry problems that may have been found by you or your staff during the course of your studies. Problems occasionally arise that may not have been obvious during routine health rounds by the veterinary staff or may have developed quite quickly (such as a flooded mouse cage) or due to a sudden change in the environmental conditions of the animal room, such as a spike in temperature or a reversal in directional airflow. Some of these problems, if undetected or unreported, could endanger the animals and your research. There should be no hesitance in contacting the veterinary staff for assistance or notifying them early of health issues or logistical problems.

When establishing and managing relationships, do not forget to include the individuals in top administrative posts that support your research in less direct—though no less important—ways. Such influential members may include your department chair, the dean of your school, and the IO.

The IO oversees the IACUC activities and evaluates the performance of the attending veterinarian (Fig. 1) [1]. The IO is ultimately (and legally) responsible for ensuring that animal research at his or her institution is conducted humanely and in compliance with all applicable regulations. It is also the IO's job to ensure that sufficient resources (mainly money and space) are allocated to the animal program to maintain or improve its quality and effectiveness. Enlisting the IO's cooperation can be invaluable in the event that you might need financial or logistical assistance, especially with startup costs, subsidization of certain research activities with high institutional priority, specialized equipment purchases, or cover of unforeseen expenses.

Other valuable relationships include the office of Contracts and Grants (sometimes called Sponsored Programs), Physical Plant or Facilities Management, Capital Programs, and the organization's security or police departments. The latter is particularly important to protect researchers and their research in the event they become the target of animal rights activists. Animal rights organizations are known to have infiltrated research laboratories or animal resource departments and post online recorded videos of lab animal-related activities. More recently, nonviolent tactics have yielded to violent terrorist acts, such as fire bombings of researchers' residences and vehicles. Because of this, it is advisable to conduct thorough

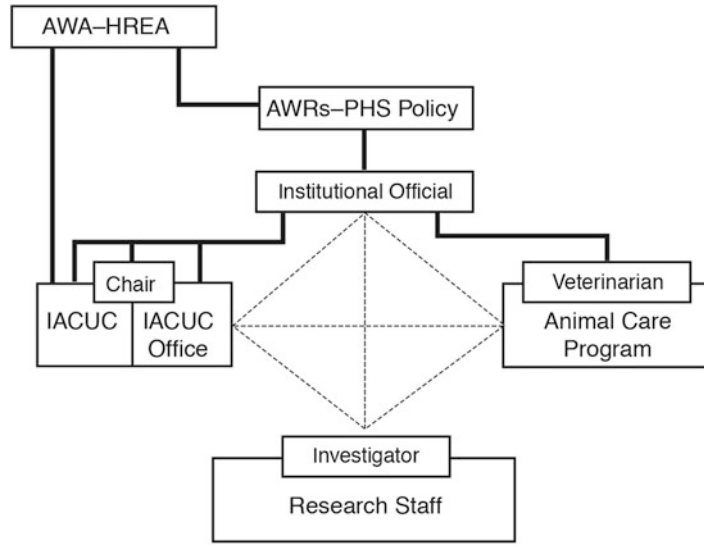

Fig. 1 Components of an animal care and use program. Heavy lines represent the mandate from the Animal Welfare Act and Heath Research Extension Act that the Secretaries of Agriculture and Health and Human Services develop guidelines for the use of animals in research and for IACUCs, and require established lines of authority from the IO to the IACUC, IACUC staff, and veterinarian. Dotted lines represent the need for cooperation and communication among components

background checks on potential lab employees—and conduct your research in strict accordance with your approved animal protocol.

5 Animal Facilities and Equipment

5.1 Housing and Procedural Space

Vivarium facilities may be centralized, decentralized, or both. *Centralized facilities* have the advantage that all activities involving animals (housing, breeding, and experimental procedures) occur within a single, secure, and environmentally controlled area. This is particularly important when working with rodents, as their health or infectious status may preclude transporting animals from one facility to another for breeding or experiments. Another advantage of a centralized vivarium is economies of scale as a result of availability of shared resources (personnel, equipment, and services). The greatest disadvantage of centralized facilities is their relative vulnerability to catastrophic losses due to power failures, natural disasters, infectious outbreaks, and unlawful animal rights activities, which may potentially decimate large populations of valuable experimental animals.

Decentralized facilities consist of multiple vivaria, typically under the management of the animal resource department. Advantages of decentralized facilities include greater protection against

catastrophic losses and, in general, added convenience for researchers, as the animal facility may be located near the investigator's laboratory. Disadvantages of decentralized facilities include less efficient use of personnel, equipment, and services as well as duplication of resources and need for additional security.

Regardless of the type of housing, it is imperative that researchers receive appropriate instruction on the humane care and use of animals, in addition to proper facility orientation and regulatory instruction and training from the animal resource staff. Specifically, researchers may be required to obtain security clearances, keys, or electronic card access to the animal facilities and must learn about applicable standard operating procedures (SOPs).

The choice of location for a vivarium or study area is also critical. In particular, the structural integrity of facilities for the housing of aquatic species must be carefully assessed, as the weight of water-filled tanks can exceed the maximum weight tolerance of the floor. For this reason (and to mitigate potential floods), most aquatic facilities are set up in the bottom floor of a building, immediately above the structure's slab.

5.2 Assignment of Vivarium Housing Space

This critical and delicate issue is typically delegated to the attending veterinarian or to the facility manager. In most instances there is good rationale for the fair assignment of space, such as degree of grant support or departmental or institutional priorities. In other cases, space is assigned on a first-come-first-serve basis. It is advisable to contact your animal resource department well in advance of planned experiments or if you anticipate an imminent or significant increase in the need for animal space, e.g., when a grant is funded or has received a high priority score. Furthermore, the attending veterinarian may be in a position to lobby on your behalf to help you secure needed support from the administration regarding justification for additional space.

5.3 Study Areas and Satellite Facilities

Two additional forms of animal housing areas have been defined: "study areas" (USDA-defined) and "satellite facilities" (PHS-defined). The Animal Welfare Regulations define "study area" as any building room, area, enclosure, or other containment outside of a core facility or centrally designated or managed area in which animals are housed for more than 12 h. The PHS Policy defines a "satellite facility" as any containment outside of a core facility or centrally designated or managed area in which animals are housed for more than 24 h. Study areas and satellite facilities are essentially "pockets" of vivarium space scattered throughout a campus or a particular building. Generally, these rooms are an extension of an individual researcher's laboratory whose own personnel look after the husbandry or breeding of the animals.

Investigator-maintained satellite facilities and study areas are particularly prevalent among research laboratories housing aquatic

species, such as zebrafish and *Xenopus* frogs, although they can also be part of specialized ("core") applications, such as surgery, imaging, or electrophysiology centers (*see* below).

Satellite facilities and study areas must conform to all federally mandated environmental standards for housing of laboratory animals, such as appropriate lighting cycles, control of temperature and humidity within acceptable ranges for the species, adequate ventilation, sanitization and disinfection methods, and animal health check schedules. Researchers who maintain study areas must be diligent to meet such standards and create and maintain documents such as SOPs, staff training records, and daily room environmental conditions and activities such as health checks, husbandry practices, and room sanitization. As well, satellite and study areas are subject to IACUC inspections at least semiannually.

In practice, IACUCs and institutional administrators, as well as regulatory agencies and accrediting bodies, typically discourage the establishment or maintenance of satellite facilities and study areas for multiple reasons, such as occupational health concerns, relatively lax security, and—frequently—inadequate veterinary oversight (*see* below under *Disadvantages*). Satellite facilities and study areas must be inspected, qualified, and approved by the IACUC prior to their use for animal housing or procedures. Though at times it might be tempting to house animals or conduct experimental procedures in unapproved locations, you must be aware that such practices are universally condemned by IACUCs and constitute a serious noncompliance reportable to both NIH/OLAW and the USDA, as applicable.

Advantages of maintaining a satellite facility or study area include the following:

1. Added researcher freedom, convenience, and flexibility of access and schedules.
2. Protection of unique, delicate, or valuable equipment.
3. Under some circumstances it may result in cost savings over central vivarium management.

Disadvantages of maintaining a satellite facility or study area include the following:

1. Decreased personal health and safety: The main occupational health risk is exposure to laboratory animal allergens (LAA). Personnel working directly with the animals (primary exposure) as well as those involved in nearby nonanimal activities (incidental or "second-hand" exposure) are more likely to become exposed to LAA in a study area setting. This is due to (a) lack of specialized protective equipment, such as change stations, laminar flow hoods, and bedding disposal units, which are typically available in regular centrally managed vivaria, and

(b) lack of or inappropriate use of personal protective equipment (PPE) or apparel that would otherwise be mandatory in regular vivaria. Additionally, personnel experiencing incidental exposure are less likely to be enrolled in institutional occupational health and safety programs or receive awareness training in this area.

2. Compromised animal health: Infrequent or less rigorous veterinary health checks of animals housed in study areas may lead to the delayed detection of sick or injured animals.
3. Compromised animal biosecurity: Insufficient or improper room sanitization or health surveillance practices can result in higher incidence of infections among laboratory animals. In addition, the usual absence of health surveillance programs in study areas can lead to delayed detection of outbreaks of animal infection or disease. Cross-traffic of research personnel between study areas and vivaria can potentially endanger the health status of the latter by sparking infectious outbreaks.
4. Lower physical security: Reduced security can potentially lead to equipment theft or cause research staff to become exposed to harassment or attacks by animal rights activists and other criminal activities.
5. Absence of economies of scale: Added equipment expenses and duplication of resources (personnel, equipment, supplies), as the provision of caging equipment (racks, cages, tanks) necessary for the housing of animals in study areas, are typically the financial responsibility of the PI, whereas in regular vivaria these expenses are covered by grant funds or are built into the per diem rates.
6. Inefficient and costly utilization of lab space: Valuable laboratory space is typically sacrificed or lost to the housing or breeding of animals. In addition, necessary physical renovations, upgrades to the ventilation system, and general maintenance of satellite facilities and study areas are the financial responsibility of the investigator. In most instances, a realistic cost-benefit analysis tends to favor housing animals in centrally managed vivaria.
7. Compliance costs: Researchers maintaining animals in study areas may incur potential additional expenses for IACUC inspections and veterinary health checks. Since these inspections are mandated by law, reduced cost-effectiveness associated with decentralization may lead to the assessment of compliance charges, which are normally built into the per diem rates in centrally managed vivaria.

5.4 Satellite Surgical Facilities

Institutions that lack a central surgical resource have little choice but to approve the establishment of researcher-maintained satellite

surgery facilities, typically within the confines of a researcher's laboratory. Even when a central surgery area may be available, investigators may successfully justify the creation of their own satellite surgery areas on the basis of their need for specialized surgical or data collection equipment that may not be available, practical, or safe to place in a centrally managed shared resource. By and large, the pros and cons listed above for satellite facilities and study areas apply also to satellite surgery facilities. One important additional aggravating factor with satellite surgeries is inability of the institutional veterinary staff to adequately monitor (if at all) the surgical manipulations, postoperative recovery, and pain management of animals undergoing surgery in remote locations. The latter may lead to institutional criticism by external regulatory or accrediting bodies.

5.5 Research Core Facilities

These are specialized areas designed to provide specific services to the institutional research community. Such facilities may be devoted to maintaining animal breeding colonies (e.g., mice, zebrafish); providing assisted reproductive technology services (creation of transgenic or other genetically manipulated animals, rederivation, embryo or gamete cryopreservation, or in vitro fertilization); imaging technologies (e.g., MRI, PET, CT scanning, optical imaging, ultrasonography); behavioral testing of rodents, fish, or nonhuman primates; surgery; metabolic and nutritional studies; irradiation; biocontainment, etc. Besides being subject to the applicable animal care and use regulations, some core facilities may be subject to additional requirements. For example, because of their potential for nefarious misuse or bioterrorism, users of irradiation facilities based on radioactive sources (e.g., cesium or cobalt) and use of microbial "select agents" are subject to background checks by the Department of Homeland Security and the FBI, biometric identification, and required to maintain extraordinary security measures, such as sophisticated alarm systems connected to a central security office or a campus police department.

6 Animals, Equipment, and Supplies

6.1 Sources of Laboratory Animals and Tissues

The quality and reliability of research data obtained from animals are closely correlated with the quality of the animals, particularly their genetic purity (i.e., inbred strains vs. outbred stocks), microbiological status, and incidence of injury or disease, as well as the integrity and consistency of the environmental conditions. Concurrent infections that may not show external signs of disease (subclinical) can still affect research results in profound ways [22, 23]. Reputable animal vendors have stringent health surveillance programs in place and generally their animals are free of specified pathogens. This is particularly true for the main rodents

and rabbit suppliers. In the rare cases when an infectious outbreak is detected in their commercial breeding facilities, they immediately contact the institutional purchasing agents, who in turn notify the attending veterinarian and researchers that may have purchased animals from a contaminated source. Because health status of aquatic species, particularly *Xenopus* frogs and zebrafish, is less well defined, the choice of suppliers becomes all the more critical. Annually published laboratory animal Buyers' Guides are good resources for information regarding animal suppliers [24, 25].

It is important to procure animals from institutionally approved sources, using proper purchasing or animal transfer channels and abiding by any Materials Transfer Agreement (MTA) restrictions. In addition, the creation or importation of genetically modified animals, usually rodents, may require registration with the Institutional Biosafety Committee (IBC) in accordance with the NIH Guidelines for Research Involving Recombinant DNA [26]. Prior to importing new animal genetic lines, it is recommended that you consult with the IBC to find out whether the desired strain of animal may already be available in your organization. This extra step may help you minimize research delays and save a significant amount of grant or departmental money.

Responsible institutions have strict animal quarantine, isolation, or rederivation requirements for the importation of animals from nontraditional sources. The goal of these mechanisms is to protect institutional animal colonies against the introduction of infectious agents. It is critical that you consult with the institutional veterinarian prior to the importation of animals from unapproved suppliers.

Tissues and biologic products of animal origin, such as cell lines, tumor fragments, and serum, may become contaminated with pathogens, which could trigger outbreaks of disease at your institution upon their inoculation into animals housed in a "clean" colony. Prior to their introduction into experimental animals, it is a good practice to have these products tested by polymerase chain reaction (PCR) for the presence of pathogens. In the modern vivarium, tissues and biological products are one of the greatest sources of concern for introduction of excluded pathogens and researchers must be diligent in their efforts to screen these products.

Tissues and biologic products of human origin may also carry pathogens that could potentially expose animals and animal workers to infection or disease. Additionally, use of primary tissues or cells from human patients may require approval from the Institutional Review Board (IRB). If you plan to work with human embryonic stem cells in animal research, be aware that the National Academies called in 2005 for the establishment of institutional Embryonic Stem Cell Research Oversight (ESCRO) committees

and provided guidelines for the management of ethical and legal concerns in human embryonic stem cell research [27].

6.2 Equipment

The conduct of responsible animal research requires that appropriate housing (racks, cages, tanks) be provided for the well-being and comfort of the animals. This will maximize reliability and reproducibility of research data. As indicated above, provision of physical space in the vivarium for housing and procedures, maintenance of proper environmental conditions, and supply of personal protective equipment (PPE) are typically the responsibility of the animal resource department in centrally managed vivaria. Conversely, these are the researchers' responsibility in investigator-maintained satellite facilities. Researchers must be aware that the goal of wearing appropriate PPE is to protect the health of both the animals (biosecurity) and the researchers and animal care personnel.

6.3 Feed, Bedding, and Water

The quality and reliability of data obtained from research animals are also closely linked to the quality of the feed, bedding, and water used. Reputable suppliers of feed and bedding offer consistent, high-quality, products. The goal is to maintain stable environmental conditions for research animals to avoid the introduction of uncontrolled variables. The Buyers' Guides mentioned above also list feed and bedding suppliers [24, 25]. Keep in mind that even high-quality feed and bedding may cause unwanted research interference. For example, seasonal variation in the phytoestrogen content in standard rodent diets can affect reproductive function in mice. Similarly, corncob bedding may be inappropriate for certain metabolic or nutritional studies in rodents where strict fasting may be necessary.

The quality of the drinking water is critical for immunocompromised animals, as even ubiquitous or opportunistic bacteria and fungi can infect the animals causing disastrous clinical or subclinical problems. Furthermore, the quality of the tank water is absolutely vital for amphibians and fish. Commercial test kits are available to measure critical parameters, such as alkalinity, hardness, pH, salinity/conductivity, chlorine/chloramines, ammonia, nitrite, and nitrates.

7 The Animal Research Environment

7.1 Protecting Researchers and Their Staff from Research Risks

As mentioned in the introduction, researchers must be fully aware of the risks involved in working with animals and enroll appropriate staff in the institutional occupational health and safety program (OHSP). Generally speaking, research institutions have broad OHSP guidelines as well as requirements that are specific to particular research or species of animal used. Requirements range from filling out a medical history questionnaire to specific vaccinations,

infectious disease, and allergy screening, and to respiratory function tests and serum banking. Typically, SOPs specific to the hazardous agent/activity are developed and must be followed. Researchers must be proactive in identifying hazards associated with their research, assisting with development of SOPs, and ensuring that they and their staff follow approved procedures. Consult with your organization's environmental and occupational health and safety representatives for specific requirements and practices.

7.2 Nonsurgical Procedures on Laboratory Animals

The most common nonsurgical techniques are injections, administration of oral substances (gavage, retrolingual), and collection of blood and other tissues or fluids. Although most nonsurgical procedures are minimally invasive and cause only slight or momentary pain or distress, it is sometimes necessary to provide anesthesia to the animals for the purpose of immobilization. It must be recognized that certain nonsurgical procedures have the potential to cause distress and are closely scrutinized by IACUCs and animal advocacy groups. Examples of these procedures are prolonged physical restraint of conscious animals, food or water restriction or deprivation, and use of "death as an endpoint," the practice of allowing animals to spontaneously die without intervening with treatment or euthanasia (sometimes employed in infectious or tumor-related survival studies).

7.3 Surgical Procedures on Laboratory Animals

The conduct of surgery on laboratory animals is subject to federal and institutional requirements that vary with the species under consideration and with the nature of the surgery. Regardless of the type of surgery, all animals must be provided adequate anesthesia at sufficient levels that will abolish consciousness and pain perception during the procedure. Animals intended to recover from the procedure (i.e., survival surgery) must be given adequate pain relief (analgesia) for the expected duration of the painful phase of the postoperative recovery. Though the length of the potentially painful period will depend on the degree of invasiveness of the procedure, as a general guideline, postoperative analgesia should be given for a *minimum* of 48 h after major surgery, or 24 h after minor surgery. However, the animal should continue to be assessed for signs of pain following this period and analgesia administered when necessary. Furthermore, the veterinarian should be consulted if surgical training is needed. Alternatively, arrangements may be made for qualified members of the veterinary staff to perform the experimental surgeries for the research lab.

Knowledge of the following definitions from the AWA and key points from the regulations will help to determine how to adequately categorize your animal protocol:

(a) *Major surgery* (major operative procedure): This is defined in the AWR as "any surgical intervention that penetrates and

exposes a body cavity or any procedure which produces permanent impairment of physical or physiological functions." Major operative procedures on USDA-covered species must be conducted only in facilities intended for that purpose (i.e., dedicated operating room) operated and maintained under aseptic conditions. Non-USDA-covered species (i.e., laboratory mouse and rat, bird, fish, and amphibian) surgery may be done in clean and temporarily dedicated areas of the lab. The veterinarian or IACUC representative should be consulted for specific guidance regarding appropriateness of the surgery areas.

(b) *Survival surgery*: Animals are intended to recover from anesthesia (i.e., regain consciousness) after the procedure. All survival surgical procedures must be conducted using aseptic technique to minimize contamination of the wound or development of systemic infection after the procedure. The indication for aseptic preparation of the skin in *Xenopus* frogs and other aquatic species is controversial, however. Chemical antiseptic agents may disrupt the normal skin flora of these species whereas surgical drapes can easily damage their delicate skin. NIH veterinarians report that the incidence of clinical complications following surgical oocyte harvesting without strict aseptic preparation is rare. On the other hand, postsurgical pain relief must always be provided unless scientifically justified by the PI and approved by the IACUC.

(c) *Non-survival surgery*: Animals are euthanized at the conclusion of the procedure *before* they regain consciousness.

(d) *Multiple major survival surgery*: Multiple surgeries *on the same animal* may be done only as an integral part of a protocol, i.e., if the two or more surgeries are related components of a single study. Multiple major survival surgeries must be scientifically justified by the PI, and approved in advance by the IACUC. Consult USDA policy #14 for details on this topic [28]. Note that although this standard originated in regard to USDA-covered species, its practice has commonly been extended to non-USDA-covered species as well.

7.4 Pain and Distress in Laboratory Animals

The recognition and alleviation of pain and distress in laboratory animals is a difficult subject to address because their expression varies greatly among the different species used in research settings. Mild-to-moderate pain in lower species, such as fish, frogs, rodents, and chickens, may be nearly impossible to recognize. For details on this subject, the reader is referred to the texts *Recognition and Alleviation of Pain in Laboratory Animals* [29] and *Recognition and Alleviation of Distress in Laboratory Animals* [30]

Because of these limitations in the proper recognition of signs of pain and distress, animals must be given the benefit of the doubt

regarding the need for pain medication. When in doubt, the best approach is to administer painkillers to the animals unless withholding such medications has been scientifically justified by the PI and approved by the IACUC. The US Government Principles IV, V, and VI address the issue of pain and distress thus [16]:

> *IV. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals.*
>
> *V. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents.*
>
> *VI. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure.*

Drugs used to prevent and relieve pain and distress in laboratory animals are typically divided into three categories: anesthetics, analgesics, and tranquilizers. A precise (and at times controversial) description of categorization is beyond the scope of this chapter; however, the reader should appreciate that there is not always a sharp delineation between categories.

Anesthetics can have local, regional, or general effects. Local anesthetics typically abolish pain perception in specific areas of the body where they are injected or applied. Regional anesthetics block specific nerves that supply larger areas of the body, which become desensitized by the injection of a local anesthetic agent. Neither local nor regional anesthetics depress the central nervous system (CNS) and therefore the animal remains conscious. General anesthetics depress the CNS leading to loss of consciousness, chemical immobilization, and lack of pain perception after injection, inhalation, or topical exposure (such as for fish and amphibian anesthesia).

Local anesthetics, such as lidocaine (Xylocaine®) and bupivacaine (Marcaine®), are injected into the skin or immediately underneath (subcutaneously) in order to numb the area and block pain sensation that might otherwise result from a surgical incision or the insertion of a large-bore needle (trocar). Local anesthetics may be applied also topically to skin or mucous membranes or injected, much in the same way dentists do on human patients. General anesthetics include agents such as isoflurane or sevoflurane inhalant gas; injectable agents, such as ketamine/xylazine combination and pentobarbital (Nembutal®); and topical tricaine methanesulfonate (MS-222). The latter is commonly used for general anesthesia of aquatic species and is applied by immersion and exposure of the animals in medicated water solution.

Analgesics (painkillers) are drugs that abolish or minimize pain perception after systemic administration, usually orally or by

injection. Common analgesics include morphine and related compounds (buprenorphine, oxymorphone) as well as drugs that have both analgesic and anti-inflammatory properties (nonsteroidal anti-inflammatory drugs, NSAIDs), such as ibuprofen (Advil®), carprofen (Rimadyl®), and meloxicam (Metacam®). Even though animals under general anesthesia do not feel pain, it is important to also administer an analgesic to control postoperative inflammation and pain upon recovery from anesthesia. It is best if the analgesic is given preemptively, i.e., before the surgical incision is made, as this will likely reduce the postoperative need for analgesia.

Tranquilizers are seldom used in laboratory animals except in large animals, mainly dogs, cats, rabbits, nonhuman primates, and farm animals. The drugs most commonly used are diazepam (Valium®), midazolam (Versed®), and acepromazine.

On occasion, the administration of analgesics or tranquilizers may interfere with the goal or outcome of the study. In these cases, researchers must present to the IACUC-detailed scientific justification for withholding pain medication subsequently to a potentially painful or distressful procedure. These studies are classified as "Pain category E"—based on the USDA annual report form 7023—and undergo extensive IACUC scrutiny [31].

7.5 Neuromuscular Blockers (NMB; Paralyzing Drugs)

The use in animal research of paralyzing drugs, such as vecuronium and curare, is a delicate matter and one that receives close scrutiny from IACUCs and veterinarians. The concern is that a paralyzed animal is unable to display signs of pain or distress—not unlike the unfortunate situation where an insufficiently anesthetized human patient wakes up during surgery but, because of the effects of the NMB, is unable to signal perception of pain.

Therefore, use of NMBs must be accompanied by the administration of general anesthetics and assurance of proper respiratory function (ventilation). In mammals, ventilation of a paralyzed animal is accomplished by endotracheal intubation and use of respirators (ventilators) or manually assisted ventilation. Use of NMBs in the absence of general anesthesia requires extraordinary scientific justification by the PI, IACUC approval, and, likely, post-approval monitoring.

7.6 Euthanasia of Laboratory Animals

Euthanasia means painless and humane killing. With few exceptions, euthanasia is administered to laboratory animals at the conclusion of a study as soon as possible after the experimental endpoint described in the research protocol has been reached. Euthanasia is also administered for humane reasons if the animals experience unanticipated pain and distress, even if the experimental endpoint has not yet been reached. The document, *Guidelines for the Euthanasia of Animals* from the American Veterinary Medical Association (AVMA) [32], is the most comprehensive resource on this subject and forms the basis for what regulatory agencies in the

United States consider acceptable. The most recent version of this document was published in 2013 and periodic revisions appear every 5–7 years. NIH also has developed guidelines on euthanasia of embryos, fetuses, and adult animals of various species and published other relevant references [19].

Although the choice of euthanasia method and agent largely depends on the animal species and the scientific goals of the project, the use of an overdose of anesthetic, such as inhalant isoflurane, injectable pentobarbital, or topical MS-222, is generally the preferred method. Less preferable is to use physical methods, such as exposure to ice water, decapitation, cervical dislocation, and pithing (although the experimental design may require a physical method). Though acceptable with conditions, exposure to high concentrations of carbon dioxide (CO_2) is controversial, due to the potentially noxious effects of CO_2 [33].

When making specific recommendations on euthanasia methodology, guidelines generally distinguish between adult and fetal or larval stages based on their degree of development of the neural mechanisms necessary for detecting noxious stimuli and perceiving pain [34]. For example, there is no evidence to indicate that zebrafish are capable of perceiving pain or distress during the first week of development. Consequently, zebrafish embryos $\leq$7 days postfertilization (dpf) may be euthanized by exposure to a bleach solution. For $\geq$8 dpf zebrafish, on the other hand, the preferable method of euthanasia is an overdose of tricaine (MS-222) by immersion for $\geq$10 min after opercular movement ends. Other acceptable methods (e.g., when the previous method interferes with data collection) include (1) immobilization by submersion in ice water for at least 10–20 min following cessation of opercular movement, (2) anesthesia with MS-222 followed by rapid freezing in liquid nitrogen, or (3) decapitation with a sharp blade [34]. The latter physical method requires scientific justification and IACUC approval.

While adult rodents are typically euthanized by an overdose of anesthetic or exposure to CO_2, in embryos and fetuses up to day 14 gestational age, neural development is minimal and pain perception is unlikely. For this reason, euthanasia of the mother (using methods appropriate for adult rodents) or removal of the embryos or fetuses from the uterus is considered acceptable as this would ensure their rapid death due to interruption of oxygen supply and lack of independent viability at this developmental stage. Fetuses at 15 days of gestation or older may have developed neural pain pathways and possibility of pain perception. Because of their diminished sensitivity to inhalant anesthetics and CO_2, however, unconsciousness must be induced by chilling by indirect contact with a wet ice slurry. Euthanasia by decapitation with sharp surgical scissors or rapid freezing in liquid nitrogen is also an acceptable method for laboratory rodent fetuses [19].

7.7 Following Protocol, Policies, and Procedures

Adherence to the research protocol as approved by the IACUC and all applicable policies, rules, and regulations is paramount. Therefore, principal investigators and staff members must be thoroughly familiar with their approved protocols. Various regulatory agencies require IO/IACUCs to submit reports regarding animal welfare activities and noncompliance, to include established recurring, standardized reports as well as on a case-by-case basis. For example, the PHS Policy establishes that the following instances must be reported to NIH/OLAW [35]:

1. Any serious or continuing noncompliance with this policy.
2. Any serious deviation from the provisions of the *Guide*.
3. Any suspension of an activity by IACUC.

Researchers must be aware that failure to comply with regulations may result in mandatory reporting to agencies outside the institution and could progress to suspension of funding, cessation of research activity, and other disciplinary actions including termination of employment at the organization.

7.8 Common Causes of Noncompliance Include

(a) *Failure to respond promptly to veterinary directives.* Institutions and regulatory agencies fully expect that researchers will respond promptly to communications from the veterinary service relative to the health and Well-being of research animals under their protocols, especially when following its directives and recommendations with regard to treatment or euthanasia of sick or injured animals. At least one veterinarian serves on the IACUC and he or she has delegated authority from the IACUC and the IO to temporarily suspend research activities if the health, welfare, or safety of the animals is at risk.

(b) *Failure to follow the approved protocol.* Be aware that even seemingly minor departures, such as failure to list staff members who work with the animals (including temporary students) or slight changes in the experimental design, can have severe repercussions and trigger lengthy and painful investigations of noncompliance during which a protocol is likely to be suspended. Not only will the suspension delay the study but can also result in exposure of the institution (and the researcher personally) to criticisms or attacks from animal rights and advocacy groups. It is essential that the IACUC administrative staff and/or veterinarian be consulted regarding planned protocol changes and IACUC approval secured before implementation.

(c) *Conducting research without IACUC approval* (absence of an approved protocol; use of an expired protocol). These violations are automatically reported to NIH or USDA, as appropriate, and usually result in lengthy suspension of animal

research privileges or placement of a principal investigator on probationary status.

It is worth noting that, in addition to the research delays, there may be financial repercussions from NIH for conducting research in a manner that violates PHS Policy, such as during protocol suspension or after protocol expiration. During the period of suspension, charges are not to be made to the grant for any research activities involving animals covered by the suspended protocol. However, funding components may allow expenditure of NIH grant funds for maintenance and care of animals on a case-by-case basis [36].

7.9 Inspection Participation and Responding to Noncompliance Reports

Frequent and effective communication with the attending veterinarian and the IACUC will avert many problems related to animal health and noncompliance issues. Many IACUCs and veterinary services publish newsletters or post regulatory and policy updates on their websites. Be sure to check these regularly. In addition, be an active participant in inspections and never attempt to mislead or deny access to authorized personnel conducting an inspection.

If you are presented with a report or an allegation of noncompliance, the best course of action is to fully cooperate with the IACUC's investigation by providing prompt and candid responses. IACUCs recognize that honest mistakes do occasionally happen and they generally take steps to handle the noncompliance in a manner acceptable to the regulatory agencies while attempting to restore the protocol to active status as soon as possible. This may not be the case if the IACUC perceives that the PI is not forthcoming with the required information, attempts to hide or distort facts, or stonewalls the investigation.

8 Conclusion

Responsible conduct of animal research is expensive, onerous, and subject to myriad restrictions and ever-present regulatory oversight. Whenever possible, researchers should make every effort to pursue or consider nonanimal alternatives (*replacement*). When these are unavailable, attention should be directed at minimizing the number of animals used (*reduction*) as well as reducing the invasiveness of the interventions to minimize pain and distress (*refinement*). Collaborations with the veterinary staff and other key members of the animal program will facilitate navigation of the regulatory maze, expedite fulfillment of training and administrative protocol requirements, streamline the overall conduct of your studies, and ensure humane care and use of research animals. When things do not go as planned and regulatory noncompliance occurs, it is best to admit errors, cooperate fully with any

investigation, be forthcoming with the requested information, and learn from the experience in order to avoid making similar mistakes in the future. Bear in mind that the regulations are designed to protect animal welfare as much as they are for the protection of personnel from occupational risks and protection of the institution from regulatory sanctions and criticism or attack from animal rights activists, including violent terrorist acts.

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Francisco J. Pelegri (ed.), *Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods*, Methods in Molecular Biology, vol. 1920, https://doi.org/10.1007/978-1-4939-9009-2, © Springer Science+Business Media, LLC, part of Springer Nature 2019

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