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Fluorescent Analysis of *Drosophila* Embryos

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CELLULAR ANALYSIS IN *DROSOPHILA* is frequently being applied to problems that were once addressed only through genetic, molecular, and biochemical approaches. A number of factors have contributed to this renaissance in *Drosophila* cell biology: Many of the procedures for cellular analysis are efficient, inexpensive, and relatively easy to perform; the generation of highly specific antibodies for immunofluorescent analysis is now routine (see Rebay and Fehon, this volume); most *Drosophila* researchers have ready access to conventional, confocal, or deconvolution fluorescent microscope systems; and finally, a variety of high-quality fluorescent probes are now commercially available (Table 9.1). More recently, the availability of green fluorescent protein (GFP)-tagged proteins has made live fluorescent analysis possible (see Hazelrigg, this volume).

The early *Drosophila* embryo is particularly amenable to fluorescent analysis. Large numbers of specifically staged embryos are easily collected from normal and mutant stocks. There is a wealth of molecular and genetic reagents that make the cellular analysis particularly powerful. The morphological and cellular events of embryogenesis have been extensively characterized (Foe and Alberts 1983; Foe et al. 1993). For example, directly after fertilization, the embryo proceeds through a series of rapid nuclear divisions that rely on the highly coordinated dynamics of the microtubules, microfilaments, and other cytoskeletal components (Warn et al. 1984; Karr and Alberts 1986; Kellogg et al. 1988). During this time, critical events that establish the axis and patterning in the embryo are occurring (for review, see St Johnston and Nüsslein-Volhard 1992). These events have been thoroughly described through fluorescent analysis and provide an excellent resource in which to analyze the primary cellular defect in newly isolated mutations.

As many of these early events are under maternal control, much of this analysis has been performed with maternal-effect mutations (Sullivan et al. 1993). With the development of the FLP-FRT (site-specific FLP recombinase–FLP recombination target) system, it is now possible to efficiently generate germ-line clones of zygotic lethal mutations and analyze the maternal effect of these mutations (Golic 1991; Dang and Perrimon 1992). Approximately 70–80% of the genes that mutate to zygotic lethality are also required in the early embryo. Consequently, this provides a general alternative approach toward examining the cellular basis of some of the more intractable zygotic lethal mutations

Table 9.1. Commercially Available Reagents

Structure	Probe[a]	Vendor/Part no.[b]
Nucleus/DNA	DAPI	Sigma/D 9542
	PI	Sigma/P 4170
	anti-histone	Chemicon/MAB052
	Hoechst 33258	Molecular Probes
	OliGreen™	Molecular Probes
Cytoskeleton		
F-actin	FITC-phalloidin	Sigma/P 5282
	TRITC-phalloidin	Sigma/P 1951
actin	anti-actin	ICN Biomedicals/clone C4
tubulin	β-tubulin	Chemicon/MAB380
	α-tubulin	Sigma/T 9026
Membrane	FITC-ConA	Molecular Probes
	anti-py99	Santa Cruz Biotechnology
	α-spectrin	Sigma/S 1390
Motors	dynein	Chemicon/MAB1618
	kinesin	DSHB/SUK4,5
Other	adducin-related protein	DSHB/1B1
	even-skipped	DSHB/2B8
	Wingless	SHB/4D4
	engrailed	DSHB/4D9
	syntaxin	DSHB/8C3
	cyclin B	DSHB/F2F4
Secondaries	FITC goat anti-mouse	Chemicon/AP124F
	FITC goat anti-rabbit	Chemicon/AP132F
	TRITC goat anti-mouse	Chemicon/AP124R
	TRITC goat anti-rabbit	Chemicon/AP132R
	Cy5 goat anti-mouse	Chemicon/AP124S
	Cy5 goat anti-rabbit	Chemicon/AP132S

[a]Abbreviations: (ConA) concanavalin A; (DAPI) 4,6-diamidino-2-phenylindole; (FITC) fluorescein isothiocyanate; (PI) propidium iodide; (py99) phosphotyrosine; (TRITC) tetramethylrhodamine isothiocyanate.
[b]DSHB is the Developmental Studies Hybridoma Bank (University of Iowa).

(Garcia-Bellido and Robbins 1983; Perrimon et al. 1984, 1989; Perrimon and Mahowald 1986). Transformants bearing GFP-tagged proteins are now frequently used for live cellular analysis (see Hazelrigg, this volume). Fluorescent analysis of fixed samples complements this approach. Fixed analysis allows many more embryos to be examined in a single session on the microscope, and the fixed preparations are stable for long periods. Antiquenching reagents in the mounting media allow extensive documentation of the samples without deterioration of the fluorescent signal (see p. 156). Double- and triple-labeling for colocalization studies are easily performed (see Figure 9.4B,C). Fixed samples also enable one to record the images over multiple planes for three-dimensional reconstructions.

This chapter describes the most common and generally applicable procedures for fixed fluorescent analysis of the *Drosophila* embryo. Pioneers in this field such as Rabinowitz (1941), Zalokar and Erk (1977), Foe and Alberts (1983), and Mitchison and Sedat (1983) laid the groundwork for many of the techniques described here. *Drosophila* embryos are protected by an outer chorion layer as well as an impermeable and opaque vitelline membrane (Figure 9.1). Consequently, preparation of *Drosophila* embryos proceeds as follows: chorion removal, vitelline membrane permeabilization, fixation, and finally, vitelline membrane removal. These procedures as well as procedures for collecting, staining, and mounting the embryos are described below.

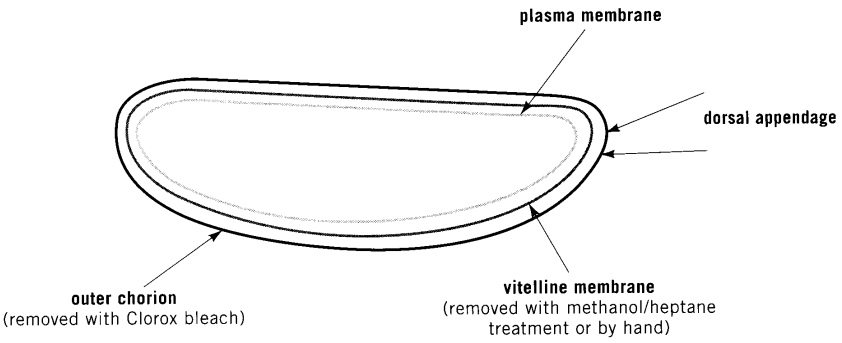

Figure 9.1. Membranes of the *Drosophila* embryo. Each embryo contains a plasma membrane, vitelline membrane, and outer chorion.

PROTOCOL 9.1*

Embryo Collection

Materials

Supplies and Equipment

Plastic fly "collection" bottle (6 ounce; Applied Scientific AS-355N)
Lids from 35 x 10-mm disposable plastic tissue-culture dishes
Screw-cap microcentrifuge tubes (1.5 ml)
Razor blades
Synthetic mesh (120-μm, 206 821 7345; Research Nets Inc., 14207 100th Ave Ne, Bothell, Washington 98011-5126. Telephone: 425-821-7345)
Small fine-haired paint brush
Squirt bottle containing H_2O

Preparation of Grape or Apple Juice Egg Plates

Plastic-ware and ingredients:

10 x 35-mm plastic petri dish lids
700 ml of H_2O (deionized)
300 ml of juice concentrate (grape or apple)
0.5 g of methylparaben dissolved in 10 ml of **ethanol**
30 g of agar (American Bioorganics 00-58010)

Add agar to the H_2O and autoclave for 40 minutes. While autoclaving, spread the tops of the petri dishes face up on a series of trays. Add methylparaben solution to juice concentrate. Quickly and thoroughly mix the concentrate into the autoclaved solution. Immediately begin dispensing media into dishes. Once the juice plates have cooled and hardened, store at 4°C. This procedure makes 1 liter of solution and yields approximately 170 juice plates.

Yeast Paste

Mix 1 g of dry yeast and 1.3 ml of H_2O to yield a paste.

CAUTION: ethanol (see Appendix 4)

*For all protocols, H_2O indicates glass distilled and deionized.

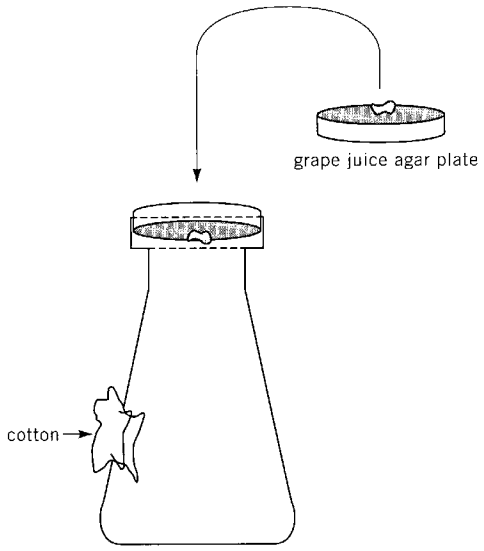

Figure 9.2. Collecting bottle and egg plate (see text for details).

Method

1. Place 200–400 flies in a 6-ounce plastic collection bottle fitted with a small cotton-filled breathing hole. Cover the bottle with a 35 x 10-mm disposable, plastic petri dish lid containing grape juice agar and a small dollop of thick yeast paste (see Figure 9.2). The eggs are deposited on the grape juice agar. Avoid opening the incubator during the egg-laying period, as the flies prefer quiet.

   Note: Typically, a single bottle is set up for each line being examined. However, if embryos of short time points (<1 hour) are being collected and/or the line does not lay many embryos, several bottles can be used.

2. While the flies are laying, prepare egg baskets as follows:

   a. Cut 1.5-ml screw-cap microcentrifuge tubes in half (see Figure 9.3). Also cut off the top of each screw cap, so that it forms an open ring that can be screwed back onto the microcentrifuge tube.

   b. Place a piece of 120-μm synthetic mesh around the threaded end of each microcentrifuge tube.

   c. Use the ring made from the screw cap to hold the mesh in place, thus creating a basket.

      Notes: If larger quantities of eggs are being fixed, larger egg baskets must be constructed. A good rule of thumb is that enough surface area should be provided so that the embryos can lie in a monolayer on the mesh.

      In general, a single basket is used for each timed embryo collection. Between collections, the egg basket is thoroughly washed with H_2O, and the mesh is replaced.

3. Obtain embryo collections at appropriate time points. Transfer the embryos from the agar plates into the egg basket using H_2O and a small paintbrush. If there are relatively few embryos, rapidly pick the embryos from the plate with forceps and transfer them in clusters to the egg basket. In either case, wash all the embryos into the mesh at the

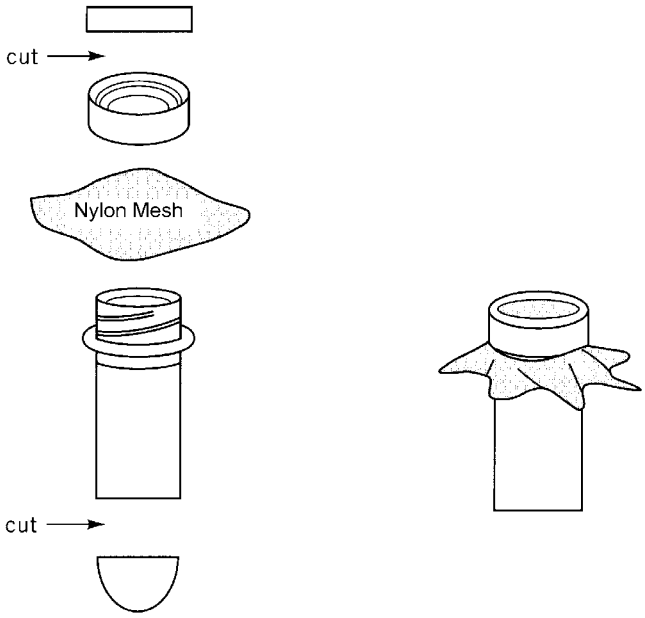

Figure 9.3. Constructing an egg basket (see text for details).

bottom of the basket using copious amounts of H_2O. Proceed rapidly with the washing to avoid anoxia.

Note: For embryo collections from various time points, dechorionation should follow immediately for each collection (see Protocol 9.2).

PROTOCOL 9.2

Embryo Dechorionation

Materials

Supplies and Equipment

Dissecting microscope
Glass petri dish
Pasteur pipette

Solutions and Reagents

Squirt bottle containing 1x Embryo Wash Solution
Bleach (Clorox)

10x Embryo Wash Solution

7% NaCl
0.5% Triton X-100
Dissolve 70 g of NaCl and 5 ml of Triton X-100 in sufficient H_2O. Adjust to 1 liter with H_2O.

CAUTION: bleach (see Appendix 4)

Method

1. Place the egg baskets in a glass petri dish partially filled with a 50% bleach (Clorox) solution (the level should be just below the rim of the basket). Use a pasteur pipette to continually rinse the embryos with the bleach solution.

   Note: Because the potency of the bleach varies, monitor the dechorionation process by observing the embryos under a dissecting microscope. When the dorsal appendages have dissolved in 80% of the embryos (~1–3 minutes), immediately proceed to step 2, below (extensive washing). Monitoring prevents damaging the embryos through overexposure to bleach.

2. Immediately wash the basket of embryos using a squirt bottle containing Embryo Wash Solution. Wash the inner edges of the basket, so that all of the embryos lie on the mesh.

EMBRYO FIXATION

Protocol 9.3 presents the following fixation procedures:

- *Formaldehyde-based Fixation Techniques.* The slow formaldehyde fix method (see Protocol 9.3, Method 1) is a modification of the Mitchison and Sedat protocol (1983) and is excellent for preserving many structures deep within the embryo because it allows time for the fix to permeate the embryo. In general, use reagent-grade formaldehyde. However, preservation of some cellular components may require higher-quality (EM-grade) formaldehyde.

  The fast formaldehyde fix method (see Protocol 9.3, Method 2) designed by Theurkauf (1992) is a modification of the slow formaldehyde fixation procedure. It preserves cortical structures and is excellent for fixing dynamic cytoskeletal structures such as microtubules.
- *Fixation with Methanol.* This is a relatively harsh procedure that destroys membranes (see Protocol 9.3, Method 3). In addition, in early embryos, the pole cells are often lost. However, it adequately preserves many structures, such as microtubules. The methanol fix has the advantage that virtually all of the embryos are devitellinized. This is important when analyzing embryos that are limited in quantity.
- *Fixation by Boiling.* The boiling fix method (see Protocol 9.3, Method 4) is often tried when more standard fixations fail. For example, immunofluorescent analysis of a number of centrosome proteins requires this fixation method. This procedure also results in devitellinization of virtually all of the embryos.
- *Fixation without Methanol (Hand Devitellinization of Embryos).* Often, the highest quality preparations are obtained by not exposing the embryos to methanol, which destroys membrane and other structures. In addition, some reagents such as fluorescently labeled phalloidin will not work if the embryos have been exposed to methanol. Foregoing the methanol, however, necessitates removing the vitelline membrane by hand. We find this procedure to be most satisfactory (see Protocol 9.3, Method 5). For examples of embryos prepared by this method, see Figure 9.4B,C.

The appropriate technique depends on a number of variables, including the preservation qualities of the cellular components being examined, the position of the components within the embryos, and the fluorescent probe used. Some general guidelines are presented, but for a new antibody or reagent, it may be necessary to try all of these techniques to determine which one is the most appropriate.

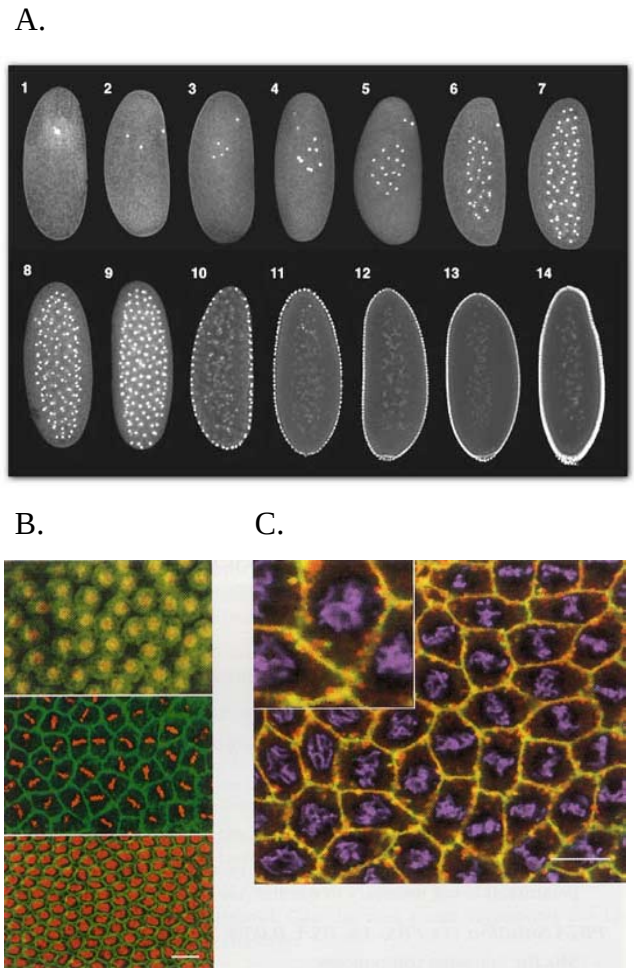

Figure 9.4. Confocal micrographs of wild-type Drosophila embryos. (A) Whole-mount Drosophila embryos stained with propidium iodide to visualize the nuclear division cycle. Shown are syncytial embryos in nuclear division cycles 1-13 and interphase of nuclear cycle 14 (cellularization). Bar, 100 Lim. (5) Surface views of Drosophila embryos prepared by hand devitellinization and double-stained for actin (fluorescein phaUoidm, green) and DNA (propidium iodide, red). Interphase actin caps (top panel) and actin-based metaphase furrows (middle panel) are shown for embryos in nuclear division cycle 13. (Bottom panel) Embryo at cellularization. Actin-based cellularization furrows surround each of the nuclei. Bar, 10 [lm. (C) Surface view of a Drosophila embryo in late metaphase/early anaphase of nuclear division cycle 13. The embryo was prepared by hand devitellinization and triple-stained for actin (fluo¬ rescein pha\lo'idm, green), DNA (propidium iodide, purple), and the furrow component, Dah (anti-Dah, Cy5-labeled secondary, red). Both actin and Dah localize to the furrows and appear to colocalize in some regions (yellow staining, inset). Bar, 10 p.m. (Inset) 2x magnification.

PROTOCOL 9.3

Embryo Fixation

Materials

Supplies and Equipment

Scissors
Glass vials (5 ml)
Pasteur pipette
Conical tube (50 ml)
3MM Whatman paper
Double-stick tape
Needle (23 gauge)
Syringe (3 ml)
Petri dish lid (35 x 10 mm)

Solutions and Reagents

Heptane
Formaldehyde (reagent and EM grade)
Methanol
Embryo Wash Solution (for preparation, see p. 145)

PEM Buffer (Karr and Alberts 1986)

0.1 M PIPES
1 mM **$MgCl_2$**
1 mM EGTA
Adjust pH to 6.9 with **KOH**.

10x PBS Solution (Sambrook et al. 1989)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

Dissolve all components in 800 ml of H_2O. Adjust the pH to 7.4 with **HCl**. Store at room temperature. It is not necessary to sterilize this solution for work with *Drosophila* embryos.

PBTA Solution (1x PBS, 1% BSA, 0.05% Triton X-100, 0.02% Sodium Azide)

Mix the following components:

10x PBS	50 ml
BSA	5 g
Triton X-100	250 µl
Sodium azide	0.1 g

Adjust volume to 500 ml with H_2O.

Preparation of Heptane Saturated with 37% Formaldehyde

1. Combine equal volumes of heptane and 37% formaldehyde in a scintillation vial (for small volumes of 2–10 ml total) or in a 100-ml glass bottle with plastic screw-top (for larger volumes of 25–80 ml total).
2. Secure the lid and shake the mixture vigorously for 15 seconds. Let the solution settle into the 2 phases.

3. Repeat this mixing procedure several (at least three) times before using the solution to ensure that the heptane becomes saturated with the formaldehyde. The saturated heptane is the upper phase in the 1:1 heptane:formaldehyde stock.

It is best to prepare the solution the day before it is to be used, shaking the vial or bottle periodically throughout the day. Because formaldehyde is sensitive to light, wrap the bottle in aluminum foil. The mixture is stored at room temperature and remains active for several months. If crystals form in the mixture upon addition of the formaldehyde, the solution will not work properly and should be discarded. Use formaldehyde from another source to prepare fresh solution.

CAUTION: formaldehyde, HCl, heptane, KCl, KH_2PO_4, KOH, methanol, $MgCl_2$, Na_2HPO_4, sodium azide (see Appendix 4)

Method 1

Slow Formaldehyde Fix

1. Remove the mesh containing the dechorionated embryos from the egg basket. The embryos should be in the center of the mesh. Cut away the excess mesh and extract excess liquid by gently blotting with a paper towel.
2. Place the mesh (embryos facing out) on the inner edge of a 5-ml glass vial. Use a pasteur pipette filled with approximately 1 ml of heptane to wash the embryos off the mesh into the vial (contaminating bits of agar will preferentially stick to the mesh).
3. Remove the mesh, and immediately add an equal volume of 3.7% formaldehyde in PEM buffer. Screw the lid on tightly and shake vigorously for 15 seconds.
4. Let the vial stand at room temperature for 20 minutes.

   Note: At the end of this period, the embryos will lie at the interface between the lower formaldehyde and the upper heptane layer. Be aware of the time, as longer fixation times result in cross-linking of the plasma membrane and the vitelline membrane and thus significantly decrease the efficiency of devitellinization.

5. Carefully remove the bottom formaldehyde phase of fluid. Use a yellow pipette tip and angle the vial to remove as much formaldehyde as possible without removing the embryos.
6. Add 1.0 ml of methanol. Cap the vial, shake vigorously for 15 seconds, and let stand for 1 minute after shaking.

   Note: Usually about half of the embryos are devitellinized and these sink to the bottom of the vial.

7. Remove the upper heptane layer, along with the embryos that did not sink. Add methanol to the embryos remaining until the vial is approximately two-thirds full. Store these embryos in methanol at 4°C.

   Note: Storing the embryos overnight at 4°C before staining allows time for clearing and often results in higher-quality images.

Method 2

Fast Formaldehyde Fix

Perform steps 1–7 as described for the Slow Formaldehyde Fix (Method 1, above). However, at step 3, add 37% formaldehyde and fix for 5 minutes at room temperature

(step 4). As with the Slow Formaldehyde Fix procedure, longer fix times decrease the yield because of poor devitellinization.

Method 3

Methanol Fix

1. Perform steps 1 and 2 of the Slow Formaldehyde Fix (Method 1, above).
2. Remove the mesh and add 1.0 ml of methanol. Cap the vial, shake vigorously for 15 seconds, and let stand for 1 minute after shaking.

   Note: Almost all of the embryos are devitellinized and sink to the bottom of the vial.
3. Remove the upper heptane layer and most of the methanol, leaving the embryos at the bottom of the vial. Add fresh methanol until the vial is approximately two-thirds full. Store these embryos in methanol at 4°C.

Method 4

Boiling Fix

1. Heat 5 ml of Embryo Wash Solution to 90–100°C in a 50-ml conical tube. Add dechorionated embryos to the heated Embryo Wash Solution.

   Note: This step causes the rapid fixation of the embryos.
2. Immediately add 40 ml of ice-cold Embryo Wash Solution and place on ice. Allow the embryos to sink to the bottom of the tube (~1 minute).
3. Use a pasteur pipette to transfer the fixed embryos in approximately 1 ml of Embryo Wash Solution to a 5-ml glass vial. Allow the embryos to sink to the bottom of the vial (~30 seconds).
4. Remove as much of the Embryo Wash Solution as possible, leaving the embryos in the bottom of the vial. Proceed with the devitellinization by adding 1 ml of heptane followed by 1 ml of methanol to the embryos. Cap the tube, shake it vigorously for 15 seconds, and let stand for 1 minute after shaking.

   Note: Almost all of the embryos are devitellinized and sink to the bottom of the vial.
5. Remove the upper heptane layer and most of the methanol, leaving the embryos at the bottom of the vial. Add fresh methanol until the vial is approximately two-thirds full. Store these embyros in methanol at 4°C.

Method 5

Embryo Fixation without Methanol (Hand Devitellinization of Embryos)

1. Wash dechorionated embryos into a 5-ml glass vial with approximately 1 ml of heptane saturated with 37% formaldehyde. Incubate the embryos in this solution at room temperature for 40 minutes.
2. Use a pasteur pipette to transfer the embryos to a small piece of 3MM Whatman paper and allow the heptane to evaporate (~30 seconds).

   Note: Ensure that the embryos stay in the neck of the pipette when transferring them. Embryos entering the body of the pipette tend to stick to the sides and are lost. Also, the embryos tend to sink quickly toward the tip of the pipette. It is best to allow them to sink and to transfer them in 1–2 drops onto the Whatman paper.

3. Transfer the embryos from the Whatman paper to double-stick tape positioned on the bottom of the lid of a small petri dish (35 x 10 mm) by gently placing the Whatman paper embryo-side down onto the tape and very gently tapping the paper until the embryos stick to the tape. Remove the Whatman paper and immediately cover the embryos with PBTA Solution.
4. Remove the vitelline membrane by hand under a dissecting scope using a 23-gauge needle mounted on a 3-ml syringe. This is best achieved by poking a small hole in the vitelline membrane at one end of the embryo and gently "pushing" the embryo out through the hole by applying pressure from the opposite end. The membrane will remain stuck to the tape and the devitellinized embryo will float up into the PBTA Solution.
5. Transfer the devitellinized embryos in PBTA Solution to a 5-ml glass vial. Store embryos in PBTA at 4°C or proceed immediately with staining of embryos (see pp. 153–156).

STORAGE OF FIXED EMBRYOS

Hand-devitellinized embryos are stored in PBTA Solution (see p. 148) at 4°C. For all other fixes, store the embryos in methanol at 4°C. Overnight storage in methanol clears the embryo and often improves image quality. However, loss of image quality does occur if the embryos are stored in methanol for extended periods of time (weeks). The extent of the deterioration depends on the probe and the antigen being examined.

REHYDRATION OF EMBRYOS STORED IN METHANOL

Embryos stored in methanol must be rehydrated before staining. Rehydration methods differ slightly for embryos fixed in methanol and embryos fixed in formaldehyde (Methods 1 and 2, below). If a new probe or antibody is being used, a gentle rehydration method is recommended (Method 3, below).

PROTOCOL 9.4

Rehydration of Embryos

Materials

Supplies and Equipment

Microcentrifuge tube (1.5 ml)
Pasteur pipette

Solutions and Reagents

Methanol
PBTA Solution (for preparation, see p. 148)
1x PBS (for preparation of 10x PBS, see p. 148)

CAUTION: methanol (see Appendix 4)

Method 1

Rehydration of Embryos Fixed in Methanol

1. Transfer the embryos, in methanol, to a 1.5-ml microcentrifuge tube. Remove as much of the methanol as possible.
2. Add 250 µl of methanol to the embryos. Gently add 250 µl of PBTA Solution, taking care not to shake the tube.

   Note: Do not shake the PBTA:methanol mixture, because bubbles that form interfere with the ability of the embryos to sink to the bottom of the tube.
3. Add PBTA Solution until the tube is two-thirds full. Invert the tube gently two to three times and let the embryos sink to the bottom of the tube.
4. Remove the solution, leaving the embryos in the bottom of the tube, and add 500 µl of PBTA Solution.
5. Allow the embryos to rehydrate in the PBTA Solution at room temperature for 15 minutes on a rotator.

Method 2

Rehydration of Embryos Fixed in Formaldehyde

1. Transfer the embryos, in methanol, to a 1.5-ml microcentrifuge tube. Remove as much of the methanol as possible.
2. Add 500 µl of PBTA Solution. Allow embryos to rehydrate in this solution at room temperature for 15 minutes on a rotator.

Method 3

Alternative Rehydration Procedure

1. Transfer the embryos, in methanol, to a 1.5-ml microcentrifuge tube. Remove as much of the methanol as possible.
2. Perform 5-minute washes in the following series of methanol:PBS solutions: 80%:20%, 60%:40%, and 20%:80%.
3. Immerse the embryos in 100% PBS, and then 100% PBTA.
4. Incubate the embryos in PBTA at room temperature for 30 minutes on a rotator.

EMBRYO STAINING

Embryos can be stained with specific fluorescent probes or antibodies through either direct or indirect immunofluorescence. Extensive washing with frequent solution changes reduces background and is key to high-quality preparations. Consequently, any extended break in the preparation (such as leaving overnight) should be made during a washing step. Washes more than 2 hours should be performed at 4°C.

Nuclear Stains

Several effective probes exist for visualizing DNA (see Table 9.1). Embryo staining procedures are described below for the following:

- Staining with propidium iodide (see Protocol 9.5)
- Staining with 4,6-diamidino-2-phenylindole (DAPI; see Protocol 9.6)

Other fluorescent DNA probes are processed as described for DAPI. Table 9.1 provides a list of commonly used probes and compatible fixations.

Antibody Staining

Unlabeled primary antibodies require the use of a labeled secondary antibody. As mentioned above, extensive rinsing and washing is the key to good preparations. A procedure for staining using primary unlabeled antibodies is given in Protocol 9.7. Variations of this procedure are discussed in Protocol 9.7 Notes.

PROTOCOL 9.5

Propidium Iodide Staining

Propidium iodide is a nucleic acid stain that is added to the mounting medium (for preparation, see p. 156; see also Figure 9.4A–C). Before staining with propidium iodide, the embryos must be treated with RNase to remove the RNA.

Materials

Solutions and Reagents

RNase (10 mg/ml)
PBTA Solution (for preparation, see p. 148)

PBS-Azide
1x PBS (for preparation of 10x PBS, see p. 148)
0.02% **sodium azide**

CAUTION: sodium azide (see Appendix 4)

Method

1. Allow the rehydrated embryos to settle to the bottom of the tube and remove as much of the PBTA Solution as possible.
2. Add enough 10 mg/ml RNase to cover the embryos and incubate at 37°C for 2 hours.
3. Remove the RNase (store this RNase at 4°C; it can be reused many times). Wash the embryos several times in PBTA.
4. Wash embryos with PBS-Azide and mount as described in Protocol 9.8. Alternatively, treat embryos with another probe or antibody.

PROTOCOL 9.6

DAPI Staining

DAPI is a commonly used DNA-binding dye. Because it is specific for double-stranded DNA, no prior RNase treatment is required. When working with DAPI, wear gloves because it is a potential mutagen.

Materials

Solutions and Reagents

PBTA Solution (for preparation, see p. 148)

100x DAPI

Dissolve 10 mg of **DAPI** in 100 ml of **methanol**. Store in the dark at –20°C.

CAUTION: DAPI, methanol (see Appendix 4)

Method

1. Allow the rehydrated embryos to settle to the bottom of the tube and remove as much of the PBTA Solution as possible.
2. Add 495 µl of PBTA and 5 µl of 100x DAPI to the embryos. Incubate on a rotator for 5 minutes. To avoid quenching, the tube must be protected from light during and after this staining step.
3. Remove the DAPI solution and discard it as hazardous waste.
4. DAPI-stained embryos require extensive rinsing. Quickly rinse the embryos three times in PBTA Solution, allowing the embryos to settle between rinses. Wash the embryos in PBTA for 1 hour. Repeat this rinse/wash cycle at least once. It is beneficial to include an overnight wash at 4°C.

PROTOCOL 9.7

Unlabeled Primary Antibody staining

Materials

Supplies and Equipment

Pasteur pipette
Microcentrifuge tubes (0.5 ml; Eppendorf)

Solutions and Reagents

PBS-Azide (see p. 153)
PBTA Solution (for preparation, see p. 148)
Primary and secondary antibodies

Method

1. Use a pasteur pipette to transfer the embryos (in PBTA) to a 0.5-ml microcentrifuge tube (Eppendorf). Allow embryos to settle to the bottom of the tube. Remove the PBTA Solution and add primary antibody (diluted in PBTA) to the embryos. (If using a new antibody, try concentrations of 0.1 µg/ml, 1 µg/ml, and 10 µg/ml.) Incubate the embryos in primary antibody at room temperature for 1 hour on a rotator (for best results, incubate the embryos at 4°C overnight).

2. Remove the primary antibody and rinse the embryos three times with PBTA, allowing the embryos to settle between rinses. Wash the embryos for at least 1 hour at room temperature. Longer washes and more rinses usually produce cleaner images. Overnight washes should be performed at 4°C.

   Note: The primary antibody usually can be reused several times and should be stored at 4°C.

3. Add fluorescently labeled secondary antibody (usually at 1 μg/ml) and incubate at room temperature for 1 hour. Make sure it is directed against the same species in which the primary antibody was generated.

   Note: Once the fluorescent secondary antibody is added, the embryos should be kept out of the light as much as possible.

4. Remove the secondary antibody. Proceed through three rinse/wash cycles as described in step 2, above.

5. At this point, either prepare the embryos for mounting (steps 6–7) or counterstain the embryos for another probe (see Protocol 9.7 Notes, Double Labeling).

6. Rinse the embryos four times in PBS-Azide to remove the detergent (Triton X-100). Proceed to Protocol 9.8.

PROTOCOL 9.7 NOTES

- *Double Labeling.* When performing a double-immunofluorescent analysis, it is possible to save time by mixing the primary antibodies together in step 1 and mixing the secondary antibodies in step 3 of the unlabeled primary antibody staining procedure (above). However, by performing the staining in sequence, the primary antibodies are not mixed and can be reused many times. To perform the staining in sequence, simply repeat the primary and secondary staining (steps 1–4). Make sure to wash the embryos thoroughly between stains to avoid contamination of the primary and secondary antibodies. The following rules apply for double labeling:
  1. When examining whether two structures colocalize, the clearest results are obtained when the separate components are stained with fluorescein and Cy5 (Figure 9.4C). Bleed-through between these channels is avoided because their emission maxima lie far apart (519 nm and 670 nm, respectively).
  2. When double staining with two primary antibodies of the same species, one of the antibodies must be directly labeled (see below).
- *Directly Labeled–Antibody Staining.* Coupling a fluorophore directly to a primary antibody is a simple procedure (Francis-Lang et al. 1999) and produces excellent images. Directly labeled antibodies produced in this way are often used when staining embryos with two antibodies generated in the same species. When counterstaining with an unlabeled primary antibody, take care to stain with the directly labeled antibody last. This ensures that the secondary used to visualize the unlabeled primary does not bind to the directly labeled antibody. To stain embryos with a directly labeled antibody, perform the following steps:
  1. Use a pasteur pipette to transfer the embryos (in PBTA) to a 0.5-ml microcentrifuge tube (Eppendorf) and let the embryos settle at the bottom of the tube.

2. Remove as much PBTA as possible and add 300–500 µl of the labeled antibody (diluted in PBTA) to the embryos.
3. Incubate the embryos in the labeled antibody at room temperature for 1 hour on a rotator in the dark.

- *Using Dyes and Fluorescently Labeled Molecules for Multichannel Labeling.* Dyes, fluorescently labeled molecules, and antibodies can be used together to generate effective double- and triple-labeled images (Figure 9.4B,C). When performing a triple-label stain, take care to remove as much bleed-through from neighboring channels as possible. If two components under investigation are closely positioned and need to be resolved, be sure to stain them with fluorophores with very different emission maxima (e.g., fluorescein and Cy5, see Figure 9.4C).

PROTOCOL 9.8

Mounting and Storage of Embryos

Materials

Supplies and Equipment

Coverslips (22 x 22 mm or 22 x 30 mm)
Glass slides
Nail polish

Solutions and Reagents

Glycerol-based Mounting Medium

For the stock, prepare 10 mg/ml 1,4-**phenylenediamine** (Aldrich P2,396-2) in 10x PBS (see p. 148). Combine 10 ml of the stock with 90 ml of glycerol. Store in 1-ml aliquots at –20°C.

Mounting Medium with Propidium Iodide

Prepare mounting medium as described above. Add **propidium iodide** to a final concentration of 1 µg/ml. Store in the dark at –20°C.

CAUTION: phenylenediamine, propidium iodide (see Appendix 4)

Method

Before proceeding with mounting the embryos, be sure that steps 6 and 7 of the procedure for unlabeled primary antibodies (see Protocol 9.7) have been completed.

1. Remove as much of the PBS-Azide solution as possible and add 40 µl of glycerol-based mounting medium to the embryos.

   Note: The mounting medium should be kept on ice, and the Mounting Medium with Propidium Iodide should be kept in the dark. Mounting medium with or without propidium iodide can be used depending on the choice of stain.

2. Transfer the embryos (in mounting medium) onto a glass microscope slide using a P-200 Pipetman with the yellow tip cut at an angle to allow pipetting of the viscous solution. Place a coverslip over the embryos and seal with nail polish.

Note: It is important that the correct amount of mounting solution (containing embryos) is placed on the slide. For example, a 22 x 22-mm coverslip requires 40 µl of mounting media. Less than this volume results in bubbles and more than this volume results in a floating coverslip that cannot be sealed with nail polish.

3. Store the slides flat at –20°C in the dark.

Note: When stored at –20°C, the embryos are stable in this medium for many days with little loss of image quality.

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