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Using 32-Cell Stage *Xenopus* Embryos to Probe PCP Signaling

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Abstract

Use of loss-of function (via antisense Morpholino oligonucleotides (MOs)) or over-expression of proteins in epithelial cells during early embryogenesis of *Xenopus* embryos, can be a powerful tool to understand how signaling molecules can affect developmental events. The techniques described here are useful for examining the roles of proteins in cell–cell adhesion, and planar cell polarity (PCP) signaling in cell movement. We describe how to target specific regions within the embryos by injecting an RNA encoding a tracer molecule along with RNA encoding your protein of interest or an antisense MO to knock-down a particular protein within a specific blastomere of the embryo. Effects on cell–cell adhesion, cell movement, and endogenous or exogenous protein localization can be assessed at later stages in specific targeted tissues using fluorescent microscopy and immunolocalization.

Keywords

Planar cell polarity; *Xenopus*; Immunofluorescence; Blastomeres; Cell movement

1. Introduction

The polarized or directional orientation of cells and the migration of cells are controlled by the planar cell polarity (PCP) signaling pathway, which is critical for many developmental processes including the apical-basal polarity of epithelial tissue, convergent-extension movements during gastrulation and neurulation, and even the orientation of inner ear sensory cells and hair follicles (1).

In vertebrates, mutations in elements of the PCP pathway can cause various developmental abnormalities affecting morphogenesis in the neural tube, kidney, heart, and sensory organs (2). Disruptions in PCP components may lead to more invasive and metastatic properties of cells, and as such, may be considered to normally exhibit tumor suppressive functions (3). Thus, PCP signaling and the functional outcomes of this signaling pathway have been areas of intense focus.

The plasma membrane of epithelial cells is a wonderful example of cell polarity that is divided into two domains. This membrane consists of the apical domain facing the external environment and the basolateral domain in contact with the internal milieu. Epithelial cells have four different physical junctional structures, tight junctions, adherens junctions, gap junctions, and desmosomes. Of particular interest, the tight junctions separate the apical and

basolateral domain in each cell, which is important to maintain cell polarity (4). The general features of cell polarity have been well defined in cultured epithelial cells (5, 6); however, polarity plays a critical role in maintaining an orderly process for proper early embryonic development and tissue morphogenesis (7, 8).

We have found the 32-cell stage *Xenopus* embryo to be a very useful system for examining how proteins that interact with the PCP pathway or represent essential components of the pathway can affect developmental processes. An advantage of this system is its well-characterized and consistent cell fate map, which allows a cell's lineage to be easily traced during experiments (9). Translation of specific endogenous proteins (affecting PCP signaling) can be inhibited, mutant proteins can be ectopically expressed in embryos with great facility and developmental effects can be examined within 2–3 days. Thus, signal transduction and differentiation processes can be assessed morphologically, histologically, as well as biochemically in a developing vertebrate. Fluorescence microscopy is an extremely useful methodology to examine the *in vivo* localization of endogenous proteins in whole organisms, and is particularly informative when examining the effects of knock-down or over-expression of specific gene products, such as cell adhesion molecules.

Using the epithelial cells of early stage *Xenopus* embryos, we have recently shown that loss-of function of proteins or overexpressing proteins that interact with Par polarity complex can disrupt cell–cell contacts and tight junctions (10). In addition to the roles of proteins in cell–cell adhesion, one can examine the role of proteins affecting PCP signaling in cell movement. Using the methods outlined here, we recently discovered how a specific protein (i.e., ephrinB1) can interact with the PCP pathway to regulate the movement of retinal progenitor cells into the eye field (11–13). Effective use of this system entails targeting specific regions within the embryos by injecting an mRNA encoding a tracer molecule along with mRNA encoding your protein of interest or an antisense MO against your protein into a specific blastomere. Effects on cell–cell adhesion, cell movement, and endogenous or exogenous protein localization can be assessed at later stages in specific targeted tissues using fluorescent microscopy and immunolocalization.

2. Materials

2.1. Equipment

1. Poly (methyl methacrylate) (PMMA) chamber (tank): Fig. 1.
2. Programmable injectors: PLI-100 Pico-Injector, Medical Systems Corp (Greenvale, NY), Narishige IM300 Microinjector (Greenvale, NY).
3. Programmable micropipette puller: horizontal puller (Greenvale, NY).
4. Stereomicroscopes.
5. Micromanipulators with mounted on a magnetic base secured to a steel plate.
6. Injection dishes: Nitex mesh (Fisher Scientific #8-670-176) on the 35 mm petri dishes.
7. Cryostat with microtome blade.

8. Nutators.
9. Superfrost slides.
10. Cover slips.
11. Fine sharpened dissecting needles.
12. Glass scintillation vials.
13. Embedding molds: Tissue-Tek Cryomold #62534-10.
14. PAP pens.
15. Slide holders.
16. Slide reservoirs.
17. Humidity chambers.

2.2. Obtaining Embryos

1. HCG: human chorionic gonadotropin made with sterile water at a concentration of 2 IU/ μ l.
2. 0.1 $\times$ MBS (Modified Barth's Solution): 8.8 mM NaCl; 0.1 mM KCl; 0.1 mM MgSO₄; 0.5 mM HEPES, pH 7.8; 0.25 mM NaHCO₃, 0.07 mM CaCl₂ in 1 l of distilled water.
3. 0.5 $\times$ MBS: 44 mM NaCl; 0.5 mM KCl; 0.5 mM MgSO₄; 2.5 mM HEPES, pH 7.8; 1.25 mM NaHCO₃, 0.35 mM CaCl₂ in 1 l of distilled water.
4. 1 $\times$ MBS: 88 mM NaCl; 1.0 mM KCl; 1.0 mM MgSO₄; 5.0 mM HEPES, pH 7.8; 2.5 mM NaHCO₃, 0.7 mM CaCl₂ in 1 l of distilled water.
5. Dejelling solutions: 2% L-cysteine in 0.5 $\times$ MBS pH to 8.1 by adding 10 N NaOH.
6. Ficoll solution: 3% ficoll in 0.5 $\times$ MBS.

2.3. Injections into a Specific Blastomere

1. mRNAs: GFP (Green Fluorescence Protein) or β -galactosidase as lineage tracers; gene of interest.
2. MOs: antisense morpholino oligonucleotides. These can be purchased with a fluorescent tag and thereby also act as a lineage tracer.

2.4. Fixation and Histochemical Reactions

2.4.1. Fixation and Histochemical Reactions for β -Galactosidase

1. 0.7 $\times$ PBS (Phosphate-buffered Saline): 0.179 g of NaH₂ PO₄ · H₂ O; 0.835 g of Na₂ HPO₄ · H₂ O; 7.154 g of NaCl in 1 l of distilled water. Adjust pH to 7.4.
2. β -Galactosidase fixative: 2% Formaldehyde, 0.2% Glutaraldehyde, 0.02% Triton X-100, and 0.01% Sodium Deoxycholate in 0.7 $\times$ PBS.

3. β -Galactosidase staining solution: 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mg/ml of X-gal (or Red-gal), 1 mM $MgCl_2$ in 0.7 $\times$ PBS.
4. MEMFA: 0.1 M MOPS (pH 7.4); 2 mM EGTA ; 1 mM $MgSO_4$; 3.7% formaldehyde.
5. Bleaching solution: 1% H_2O_2 ; 5% formamide; 0.5 $\times$ SSC (standard saline citrate).

2.4.2. Fixation, Embedding, and Sections for GFP

1. 1 $\times$ PBS: 0.256 g of $NaH_2PO_4 \cdot H_2O$; 1.194 g of $Na_2HPO_4 \cdot H_2O$; 10.22 g of NaCl in 1 l of distilled water. Adjust pH to 7.4.
2. GFP fixative: 4% Paraformaldehyde, 0.9 g of NaCl, 40 ml of 0.2 M Na_2HPO_4 , 10 ml of 0.2 M NaH_2PO_4 in 100 ml of 1 $\times$ PBS.
3. OCT compound: Tissue-Tek #4583.
4. Vectashield: Vectorlabs #H-1400.
5. Nail polish.

2.4.3. Fixation, Embedding, Sections, and Antibodies Staining for Immunofluorescence

1. 0.1 $\times$ MBS (Modified Barth's Solution): 8.8 mM NaCl; 0.1 mM KCl; 0.1 mM $MgSO_4$; 0.5 mM HEPES, pH 7.8; 0.25 mM $NaHCO_3$ in 1 l of distilled water.
2. Dent's fixative: 4 volumes of methanol; 1 volume of dimethyl sulfoxide (DMSO).
3. 1 $\times$ PBS.
4. 15% Gelatin/sucrose solution: 16.67 ml of fish gelatin (45% stock); 7.5 g of sucrose in 50 ml of distilled water.
5. Sodium azide: 10% (w/v) stock.
6. OCT compound: Tissue-Tek #4583.
7. Acetone.
8. Image-iT signal enhancer: Invitrogen #I136933.
9. Blocking solution: 1% BSA; 5% heat-inactivated lamb (or goat) serum in 1 $\times$ PBS.
10. Primary antibodies: Most common primary antibodies to show cell polarity in *Xenopus* embryos are ZO-1 (Invitrogen #61-7300), Cingulin (Invitrogen #36-4401), P-E-cadherin (Epitomics #2239-1), β -catenin (Santa Cruz #sc-7199), PKC- ζ (Santa Cruz #sc-216), etc.
11. Secondary antibodies: Most common secondary antibodies used in *Xenopus* embryos are Cy3-conjugated donkey anti-rabbit IgG (ImmunoResearch Lab #711-165-152), FITC-conjugated goat anti-mouse IgG (Invitrogen #62-6511), etc.
12. Vectashield with DAPI: Vectorlabs #H-1200.
13. Nail polish.

3. Methods

3.1. Obtaining Embryos

1. Obtain fertilized embryos: Natural mating is the most effective method for obtaining the most symmetrically and orderly cleaving embryos at 32-cell stage. For this method, both male and female frogs are primed by hormone injections. Typically, males receive an injection of 50 IUs of HCG and females receive an injection of 1,000 IUs 16 h before the experiment. Place the male and female frogs in a 30 l tank filled with 16 l of 0.1× MBS 12 h prior to the time when fertilized eggs are desired (see Note 1). The bottom of the tank should contain square petri dishes covered with a stiff plastic screen. The frogs should be left in the dark (we drape the chamber with black cloth) for the next 24 h. As eggs are laid, they drop through the plastic screen into square petri dishes, and can be collected throughout the day. We use a specially constructed acryl chamber for this purpose (Fig. 1).
2. Remove the jelly coats from fertilized eggs that have just begun to cleave by gently swirling the eggs in 4 volumes of dejellying solution (L-cysteine in 0.5× MBS) for 4–5 min. After dejellying, immediately wash embryos five times with 0.5× MBS.
3. Transfer embryos to fresh 3% ficoll/0.5× MBS in a clean petri dish. Store at 13, 18, and 23°C until they reach the 32-cell stage.

3.2. Identifying Specific Blastomeres

If the study requires localization of the marker to specific tissues or regions, it is essential to identify the specific blastomere that will give rise to that tissue. According to the fate maps constructed by Dale and Slack (14) and Moody (9), one can identify a specific blastomere with distinct developmental fates in 32-cell stage embryos. In fertilized eggs, the dorsal side of the embryo can be predicted very accurately (>90%) by noting the orientation of the first cleavage furrow. At fertilization, pigmentation of the animal hemisphere begins to contract towards the sperm entry point on the ventral side, causing the dorsal equatorial region to become less pigmented. If the first cleavage furrow bisects this lighter area equally between the two daughter cells, then that lighter area can be used as the indicator of the dorsal side, and the first cleavage furrow will indicate the midsagittal plane (15, 16). Accordingly, individual blastomeres are designated as D (dorsal side) or V (ventral side) when the embryo cleaves to 4 cells. Numbers are added to the blastomere's nomenclature as the embryo further divides; these numbers indicate the cell's position, which in turn predicts which specific tissue or region it will form. For example, in the cell movement assay in which we found that our protein of interest (i.e., ephrinB1) co-opts the PCP signaling pathway via the scaffold protein Dishevelled to encourage retinal progenitor cells to move into the eye field (12), we targeted the D1.1.1 (also called A1) blastomere, which is a major contributory cell to the retinal field (~50%) later in development. In contrast, another blastomere V1.1.1 (also called A4), which is a non-contributor (<1%) to the retinal field is normally fated to move into and populate head and trunk epidermis. Since ephrinB1 RNA can cause a portion of the V1.1.1 progeny to disperse into the eye field and populate retina, this allowed us to test

¹Alternatively, 10% Steinberg's or 0.1× MMR solution can be used for natural mating.

ephrinB1-driven cell movement and whether PCP signaling was critical for this event, and whether modulators (i.e., FGFR) can regulate this activity (Fig. 2 ; 11–13).

3.3. Injections into a Specific Blastomere at 32-Cell Stage Embryos

Before starting a specific blastomere injection at 32-cell stage embryos, careful titration of all mRNAs should be done. Two commonly used mRNA tracers are β -galactosidase and Green Fluorescent Protein (GFP). Both proteins are derived from non-vertebrates (β -galactosidase from bacteria and GFP from jellyfish), can be distinguished from endogenous vertebrate proteins, are too large to diffuse through gap junctions and have no known deleterious effects on developing vertebrate cells. However, excessive amounts of GFP, β -galactosidase or the other mRNAs or MOs (morpholino antisense oligonucleotides) can cause non-specific side effects to embryos. Therefore, one needs to carefully test for the optimal concentration of the reagent to be injected. According to our experiments, generally 100–250 pg of GFP or β -galactosidase mRNA provide a strong signal and avoid non-specific side effects. For test mRNAs or MOs, titration should be done before every experiment and injection volumes should be kept to 1–2 nl per blastomere to avoid damaging the cells. For example, to examine the role of canonical and non-canonical Wnt signaling in cell movement, epistasis experiments using dominant-negative TCF3 mRNA (DN-Tcf3; a transcription factor that associates with β -catenin to activate canonical Wnt target genes) and an MO to Daam1 (a protein with Formin homology that binds to Xdsh and activates the small GTPase Rho required by the PCP pathway) can be used. Introduction of DN-Tcf3 mRNA into a particular blastomere at levels that inhibit Wnt3a-induced secondary axes should be used. In our case, this amount is 300 pg, but each mRNA should be titrated for an appropriate effect in a two-cell embryo injection. Daam1 MO (5'-GCCGCAGGTCTGT CAGTTGCTTCTA-3') was introduced at 15 ng and found to cause PCP-associated defects (12).

3.4. Fixation and Histochemical Reactions

3.4.1. Fixation and Histochemical Reactions for β -Galactosidase

1. Harvest embryos at stage 12.5 (17) and put them in glass scintillation vials (see Note 2).
2. Rinse the embryos with 0.7× PBS.
3. Fix the embryos for 1 h at room temperature with freshly made 0.7× PBS containing 2% Formaldehyde, 0.2% Glutaraldehyde, 0.02% Triton X-100, and 0.01% Sodium Deoxycholate (see Note 3).
4. Rinse the embryos twice with 0.7× PBS.
5. Stain the embryos at 30°C in 0.7× PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal (or Red-gal), 1 mM $MgCl_2$ (see Note 4).

²At stage 12.5, D1.1.1 clones are broadly dispersed across the dorsal animal quadrant, which includes the retinal field, whereas V1.1.1 clones remain on the ventral side (18). Changes in these positions can be detected by the lineage tracers, and thereby the effects of the test mRNA on the early movements of retinal progenitor cells during gastrulation can be monitored.

³Alternatively, embryos can be fixed at 4°C overnight.

⁴Staining time can vary from 30 min to 2 h. Do not exceed 2 h, since it causes too strong background staining.

6. When staining is complete, refix the embryos in MEMPFA for 1 h at room temperature.
7. If the color of staining is difficult to distinguish because of the natural pigmentation of embryos, add bleaching solution to eliminate the pigments. Incubate stained and refixed embryos in the bleaching solution for 1 h at room temperature under a fluorescent light (see Note 5).
8. Check β -galactosidase staining under the stereomicroscope, otherwise stained embryos can be stored in the dark at 4°C for 1 day (see Note 6).

3.4.2. Fixation, Embedding, and Sections for GFP

1. Harvest embryos at stage 37–38 (17) and put them in glass scintillation vials (see Note 7).
2. Rinse the embryos with 1× PBS.
3. Fix the embryos for 1 h at room temperature with freshly made 100 ml of 1× PBS containing 4% Paraformaldehyde, 0.9 g of NaCl, 40 ml of 0.2 M of Na_2HPO_4 , 10 ml of 0.2 M of NaH_2PO_4 (see Note 8).
4. When embryos are ready for further processing, they should be rinsed three times in 1× PBS for 10 min.
5. Transfer the embedded embryos with small amount of 1× PBS into embedding mold.
6. Remove all residual PBS from embedding mold (see Note 9).
7. Immediately add OCT compound into the embedding mold (see Note 10).
8. Orient the tadpoles vertically with sharp dissecting needle under the microscope (Fig. 3b; see Note 11).
9. Freeze on dry ice with methanol for 15–30 min until OCT compound completely hardens (see Note 12).
10. Remove the block from the embedding mold (see Note 13).
11. Attach the block to the cryostat chuck using small volume of OCT compound (see Note 14).

⁵Bleaching time can vary from 1 to 4 h. Do not exceed 4 h, since it can damage the embryos. If embryos need more bleaching, transfer them to new bleaching solution and bleach only for 10 min more.

⁶For best results, capture images of the embryos immediately because the signal may decrease very rapidly after 1 day.

⁷At stage 37–38, D1.1.1 clones are nicely localized to the retinal field, whereas V1.1.1 clones are confined to the head and trunk epidermis.

⁸Alternatively, embryos can be fixed at 4°C overnight.

⁹All liquid must be removed completely before adding OCT compound, otherwise any residual buffer may cause thawing of the block when the block is touched by hand.

¹⁰If embryos are exposed to air, they may be damaged.

¹¹When embryos are sectioned horizontally, the sections may not be symmetrical between left and right sides.

¹²Care must be taken when embedding mold is on dry ice/methanol, since this system is extremely cold.

¹³This is easier when the block is still cold and very hard. There is no need to trim the block. Keep it frozen on dry ice.

¹⁴Put a couple of drops of liquid OCT compound on the chuck, place the still frozen block onto it and then transfer immediately to the cryostat.

12. Equilibrate the block at least for 30 min in the cryostat.
13. Cut 16 μ m sections at -17°C of object temperature, -25°C of chamber temperature.
14. Gently stretch out the cut sections with brush while on the cryostat so they remain cold, and place glass slide over sections and they will transfer to slide within a second (see Note 15).
15. Dry the slide (laying down) at room temperature for 30 min.
16. Mount the slides with a couple of drops of Vectashield (see Note 16).
17. Apply the cover slips onto the slides (see Note 17).
18. Seal the slides/cover slips with nail polish.
19. Check GFP under the fluorescence microscope, otherwise mounted sections can be stored in the dark at -80°C .

3.4.3. Fixation, Embedding, Sections, and Antibodies Staining for Immunofluorescence

1. Harvest embryos at stage 10.5, 15, or 37–38 (17) and put them in glass scintillation vials (see Note 18).
2. Remove all the $0.1\times$ MBS.
3. Fix embryos in cold Dent's fixative (4 volumes of methanol, 1 volume of DMSO) overnight at -20°C or for 2 h at room temperature (see Note 19). After fixing the embryos, fixed embryos should be placed at -20°C until the next steps are ready.
4. When embryos are ready for further processing, they should be rinsed three times in $1\times$ PBS for each 10 min.
5. Remove all the residual $1\times$ PBS from glass scintillation vials.
6. Embed embryos in 15% cold fish gelatin/sucrose solution for 24 h at 4°C or for 2 h at room temperature. The embedded samples can be stored for up to 2 weeks at 4°C when 0.02% Sodium azide added (see Note 20).
7. Pour the fresh 15% cold fish gelatin/sucrose solution into embedding mold.
8. Transfer the embedded embryos into embedding mold.
9. Orient the embryos vertically with sharp dissecting needle under the microscope (Fig. 3a; see Note 21).

¹⁵Waiting too long to transfer the section may result in the section rolling up.

¹⁶Vectashield can reduce the background fluorescence as well as allow clear sample fluorescence.

¹⁷Care must be taken when applying the cover slip as the frozen sections are very fragile. Avoid air bubbles and do not press the cover slip too hard, as both actions will smash the sections.

¹⁸At appropriate stages, we can check the localization of cell polarity-related proteins, such as aPKC and E-cadherin. The time at which the embryos should be harvested is based on the purpose of each experiment.

¹⁹If fixing at -20°C , all liquids must be removed completely by performing a couple of washes with cold Dent's fixative before placing at -20°C , otherwise any residual water may freeze.

²⁰Sodium azide has to be added only if the fish gelatin is stored for a long time.

10. Freeze on dry ice with methanol for 15–30 min until the gelatin/sucrose solution completely hardens (see Note 22).
11. Remove the block from the embedding mold (see Note 23).
12. Attach the block to the cryostat chuck using small volume of OCT compound (see Note 24).
13. Equilibrate the block at least for 30 min in the cryostat.
14. Cut 10 μ m sections at -17°C of object temperature, -25°C of chamber temperature.
15. After making five cut sections, the sections should be immediately transferred to polylysine-coated glass slides (see Note 25).
16. Dry the slide at room temperature for 30 min in the fume hood.
17. Store slides at -70°C until they are ready for further processes.
18. Dry the slides at room temperature for 1 h in the fume hood.
19. Dip the slides in acetone for 1 min (see Note 26).
20. Dry the slides at room temperature for 10 min.
21. Draw a boundary line around the slide edges using the PAP pen (see Note 27).
22. Transfer the slides to a reservoir of $1\times$ PBS and quickly rehydrate the slides.
23. Incubate the slides horizontally with Image-iT signal enhancer for 30 min at room temperature in a humidity chamber (Fig. 4; see Note 28).
24. Wash the slides three times with $1\times$ PBS.
25. Incubate the slides with blocking solution horizontally for 30 min at room temperature in a humidity chamber.
26. Decant all the blocking solution from the slides.
27. Incubate the slides horizontally overnight at 4°C or 2 h at room temperature, with the primary antibody diluted in blocking solution in humidity chamber (see Notes 29 and ³⁰).
28. Decant all the antibody solution from the slides.
29. Return the slides to slide holder and wash three times for 10 min each in $1\times$ PBS.

²¹This is much easier when the gelatin/sucrose solution is cooled on ice, as it becomes more viscous.

²²Once frozen, the samples must be sectioned on the same day, ideally within 4–5 h.

²³This is easier when the block is still cold and very hard. There is no need to trim the block. Keep it frozen on dry ice.

²⁴Put a couple of drops of liquid OCT compound on the chuck, place the still-frozen block onto it and then transfer immediately to the cryostat.

²⁵Waiting too long to transfer the section may result in the section rolling up.

²⁶Place the slides in a slide holder and place it in a reservoir containing acetone for 1 min. Do not exceed 1 min.

²⁷This stops liquid running off the edges of the slides.

²⁸Image-iT signal enhancer can reduce the background fluorescence as well as allow clear sample fluorescence.

²⁹Alternatively, the slides can be incubated for 2 h at room temperature.

³⁰The individual slide is covered with 0.2 ml of diluted antibody solution. The dilution factor for each antibody is different. We typically use a 1:200 dilution.

30. Incubate the slides with blocking solution horizontally for 30 min at room temperature in a humidity chamber.
31. Decant all the blocking solution from the slides.
32. Place the slides in the humidity chamber and cover each with appropriate secondary antibody and incubate slides for 2 h at room temperature in humidity chamber (see Notes 31 and ³²).
33. Return the slides to slide holder and wash three times for 15 min with 1× PBS at room temperature.
34. Dry the slides at room temperature for 1 min.
35. Mount the slides with a couple of drops of Vectashield with DAPI (see Note 33).
36. Apply the cover slips onto the slides.
37. Seal the slides/cover slips with nail polish.
38. Check the immunofluorescence under the fluorescence microscope, otherwise mounted sections can be stored in the dark at −80°C.

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³¹Add 0.2 ml of appropriate secondary antibodies to cover the sections. The dilution factor for each antibody is different. We typically use a 1:400 dilution. Do not apply secondary antibodies at a concentration greater than 1:400 since it causes high background fluorescence.

³²This and all subsequent steps must be performed in the dark.

³³Care must be taken when applying the cover slip, as the frozen sections are very fragile. Avoid air bubbles and do not press the cover slip too hard, as both actions will smash the sections.

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Fig. 1.
Schematic diagram for PMMA chamber. Cartoon depicting side view of the chamber needed for natural mating.

Fig. 2.

Experimental scheme for a specific blastomere injection. Animal pole view of a 32-cell embryo. *Lighter blastomeres* depict the dorsal side and *dark blastomeres* depict the ventral side. D1.1.1 is also called A1; V1.1.1 is also called A4. Modified from Nieuwkoop and Faber developmental series (17).

Fig. 3. Experimental schemes for sectioning. *Arrow* depicts direction of sections at early embryo ((a); stage 10.5) and tadpole ((b); stage 37–38). Modified from Nieuwkoop and Faber developmental series (17).

Fig. 4. Schematic diagram for humidity chamber. Cartoon depicting top view of the humidity chamber needed for antibody staining.