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Confocal Microscopy on *Xenopus laevis* Oocytes and Embryos

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1. Introduction

There are many problems affecting microscopy in *Xenopus*. *Xenopus* oocytes and embryos are fragile and lack connective tissue. They are also full of yolk platelets, which prevents frozen sectioning because the yolk crystallizes and tears the sections. In addition, the yolk autofluoresces, making whole-mount immunocytochemistry possible, but difficult due to background from out-of-focus fluoresecence. All of these problems can be solved through confocal microscopy. Optical sectioning eliminates the need for manual sectioning and makes background fluorescence and autofluorescence negligible. It is still difficult to image the deep structures within the embryo, but thick wax sections can be cut and confocal microscopy again applied.

For years the field of cell biology has relied heavily on microscopy to study cellular organization and function. Most microscopic techniques, however, were optimized for small or flattened cells. More recently, the advent of confocal microscopy ***(1)*** has granted cell and developmental biologists the luxury of analyzing large cells or embryos, that have been fluorescently labeled. In tissues or embryos fluorescence from outside the focal plane can completely cover the details of interest by reducing image sharpness and contrast. Thus many embryos were previously impervious to immunofluorescent microscopy except through time-consuming and sometimes destructive procedures of embedding and thin-sectioning. The confocal microscope diminishes out-of-focus information and enhances image contrast by illuminating and then detecting an identical small region of the sample. Confocal microscopes are capable of "optically" sectioning through

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large cells, small embryos, or parts of larger embryos, by collecting a series of images from various focal planes of the specimen. Individual captured images can then be compiled to construct a detailed three-dimensional view of the specimen. We have used confocal laser-scanning microscopy quite successfully to analyze the distribution of organelles, proteins, and filaments in the *Xenopus laevis* oocyte and developing embryo. This chapter will detail procedures, some of which have been adapted and modified from the work of David L. Gard and others ***(2–4)***, and some of which we have devised, but all with the goal of imaging the cellular and molecular complexities of development.

Preparation of specimens for fluorescent antibody staining and confocal imaging in *Xenopus* always begins with specimen fixation. Most importantly the fixative used needs to be compatible with the specific antibodies or stain being used in the procedure. This must be determined experimentally for individual antibodies. For example, a vimentin antibody we use works only with 2% trichloroacetic acid as a fixative ***(5)***, whereas an anti-cytokeratin antibody works with ethanol ***(5)***. Another major concern during fixation is the preservation of cellular structures of interest. Much of our work involves imaging microtubules that are best preserved using the crosslinking agents formaldehyde and glutaraldehyde. Because of these factors, choosing a suitable fixation protocol is important. This problem becomes compounded when more than one antibody is being used so different options should be tried until staining is optimized. Lastly, owing to the large size of *Xenopus* oocytes and embryos (~1.2 mm diameter) almost all fixes require at least 2 h for adequate penetration.

Our laboratory has been utilizing two types of sample preparation to take advantage of confocal microscopy. We either process oocytes and embryos as whole-mounts or use the more traditional procedures of embedding and sectioning, but the samples cut as "thick" sections of about 30 μm (rather than the usual 5–10 μm) and are then optically sectioned under the confocal microscope. There are advantages and disadvantages to both of these techniques and the time commitment is about equal.

Preparing embryos for thick sectioning requires that after fixation they are embedded in wax, a process that may decrease antigenicity. Samples are then sectioned rather easily at 30-μm intervals, but must be affixed to specially prepared gelatin-coated slides. Standard antibody incubations of only a couple of hours can be performed, because we are working with sections and not whole embryos. Fluorescence outside the focal plane in these thick sections would be a problem using an epifluorescent microscope, but is eliminated during confocal microscopy. By cutting serial

sections the distribution of a particular antigen can be analyzed through an entire embryo.

The major advantage of whole-mount immunocytochemistry on oocytes and embryos is to avoid embedding and sectioning specimens. However, because *Xenopus* oocytes and embryos are so large, long antibody incubations are necessary to achieve adequate penetration. Penetration of antibodies is indeed the limiting factor during whole-mount immunofluorescence, as reports suggest that antibodies penetrate a maximum of 150 μm into fixed *Xenopus* oocytes during an overnight incubation ***(4)***. Likewise, washes following antibody incubation need also be very lengthy. We have improved incubation conditions by routinely bisecting oocytes and embryos after fixation and prior to incubations, and including detergent in fixatives and buffers. As with thick sections, fluorescence outside the focal plane is substantially reduced using confocal microscopy. Whole-mount technology and confocal microscopy have allowed us to examine the pattern of germ plasm movement in the embryo (**Fig. 1**), as well as analyze the three-dimensional filamentous microtubule network associated with the germ plasm (**Fig. 2**) ***(6)***.

For effective observation under confocal microscopy the two sample preparations are treated differently. Thick sections on slides are mounted in an aqueous medium of phosphate-buffered saline (PBS) and glycerol. Whole-mount embryos are immersed in a clearing solution. Because early *Xenopus* embryos are filled with yolk and are absolutely opaque, a method was devised by Murray and Kirschner using a mixture of benzyl alcohol and benzyl benzoate (1:2 v/v) (Murray's Clear) to make embryos transparent. This technique has been used for fluorescence microscopy of the superficial cytoplasm of early-stage whole-mount embryos ***(7)***. Bisected or whole embryos can then be mounted in glass cavity slides or in aluminum slides with bored holes and coverslips on either side to view from the top or bottom.

2. Materials

2.1. Thick Section Immunocytochemistry

2.1.1. Embedding and Sectioning Oocytes and Embryos

1. Fixative (*see* **Note 1**)
2. 4-mL glass vials
3. 100% Ethanol
4. 39°C incubator or warming receptacle
5. Embedding wax [poly(ethylene glycol) distearate + 1% w/w cetyl alcohol]. Once mixed the wax can be stored at 39°C for up to 2 weeks.

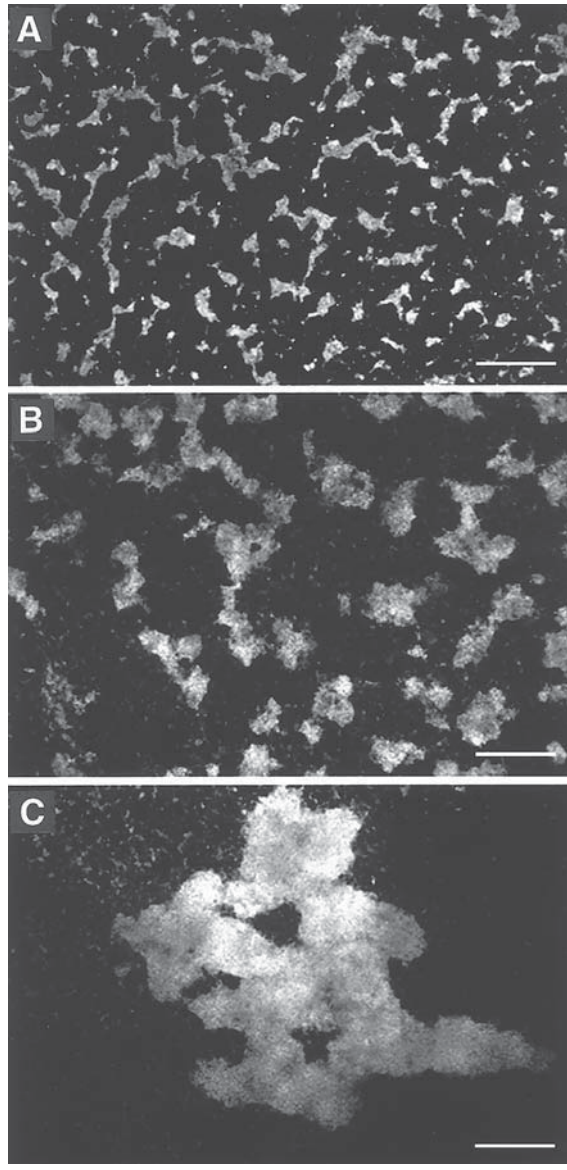

Fig. 1. Progression of germ plasm aggregation in *Xenopus laevis* prick-activated oocytes as viewed with confocal microscopy. The view is of a region of the vegetal hemisphere of the oocyte. Germ plasm is stained with chloromethyl X-rosamine (MitoTracker, Molecular Probes, Inc.), a vital dye for mitochondria found packed in the germ plasm. **(A)** Germ plasm prior to aggregation; **(B)** Germ plasm after 2 h of aggregation; **(C)** Germ plasm that has completely aggregated 4 h after prick activation. Scale bars = 25 μm.

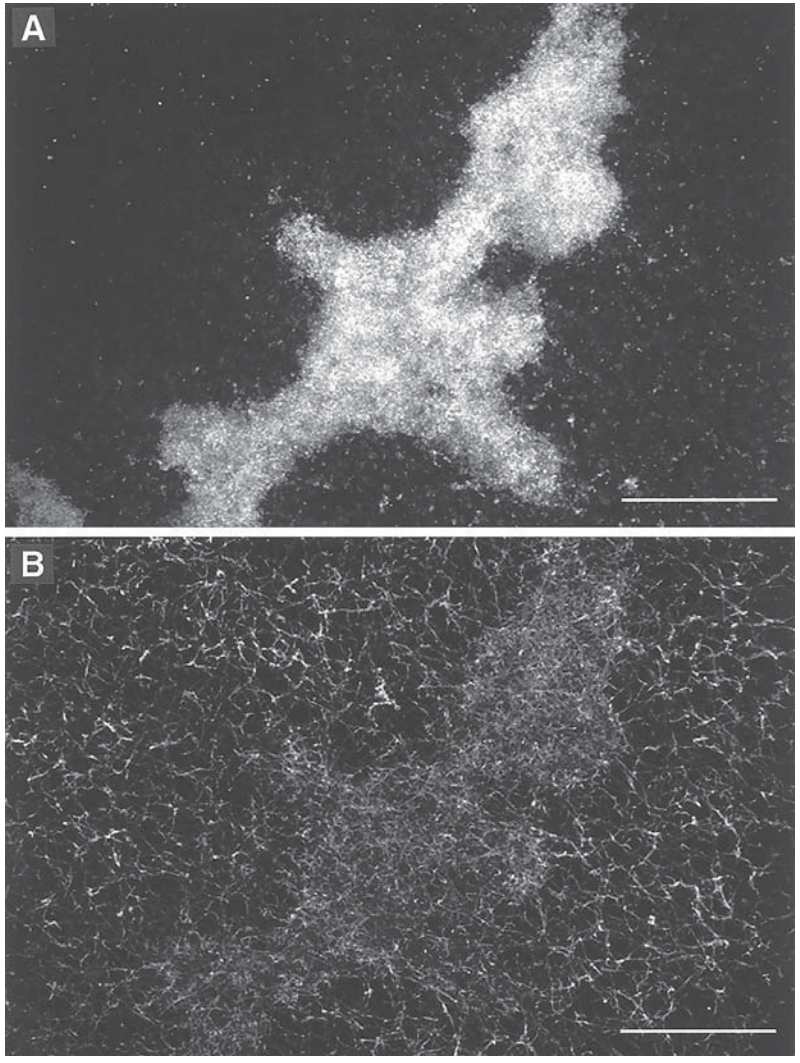

Fig. 2. Microtubules are closely associated with aggregating germ plasm. Microtubules in oocytes were whole mount labeled with an anti-α-tubulin antibody (DM1A, ICN Biomedical) subsequent to germ plasm staining. **(A)** Aggregated germ plasm stained with chloromethyl X-rosamine and viewed with confocal microscopy. **(B)** In viewing only the microtubule network, the location of the aggregated germ plasm can be predicted by a denser network of microtubules. Scale bars = 40 μm.

6. Plastic embedding trays
7. Microtome
8. Distilled water

9. Gelatin-coated slides (*see* **Note 2**)
10. Hot plate
11. Desiccator

2.1.2. Staining Thick Sections

1. Acetone series: 100%, 90%, 70%, 50% (v/v in distilled water)
2. PBS: To make a 10× stock dissolve 80 g of NaCl, 2.0 g of KCl, 14.4 g of Na_2HPO_4, and 2.4 g of KH_2P0_4 in 800 mL of distilled water and adjust pH to 7.4 with HCl. Add water to 1 L and autoclave. Dilute 10-fold with distilled water for use.
3. Horse or goat serum. Store frozen at –20°C in 10 mL aliquots.
4. Blocking buffer (10% serum, 4% bovine serum albumin in 1× PBS). Store frozen at –20°C in 1 mL aliquots.
5. Primary antibody
6. Fluorescently conjugated secondary antibody
7. Wet box
8. Eriochrome black (1:100 dilution of 1% stock in water or PBS)
9. Distilled water
10. Aqueous mounting medium made of 70–90% glycerol in 1× PBS with antifade agents (*see* **Note 4**). Store wrapped in foil.
11. Confocal microscope

2.2. Whole-Mount Immunocytochemistry

1. Fixative (*see* **Note 1**)
2. 4-mL glass vials
3. 10% Hydrogen peroxide in ethanol or methanol (optional) (*see* **Note 5**)
4. PBS
5. Ethanol and methanol
6. Fine forceps
7. Pasteur pipets
8. Scalpel
9. Petri dishes
10. 100 m*M* $NaBH_4$ in 1× PBS (optional) (*see* **Note 6**). Make fresh for each use.
11. TBSN: 1× Tris-buffered saline + 0.1% Nonidet P-40 (NP-40). To make a 10× stock of TBS dissolve 80 g of NaCl, 2.0 g of KCl, and 30 g of Tris base in 800 mL of distilled water and adjust pH to 7.4 with HCl. Add distilled water to 1 L and autoclave. Dilute 10-fold with distilled water for use and add 0.1% (v/v) NP-40. Solution can be stored at 4°C for up to a year.
12. Rocker or nutator.
13. 2-mL glass vials (1.5-mL Eppendorf vials can also be used).
14. Primary antibody diluted in TBSN + 2% bovine serum albumin (BSA).
15. Fluorescently conjugated secondary antibody diluted in TBSN + 2% BSA.
16. Cavity slides.
17. Murray's Clear Benzyl benzoate/benzyl alcohol (1:2 v/v).

18. Nail polish
19. Confocal microscope

3. Methods

3.1. Thick Section Immunocytochemistry

3.1.1. Embedding and Sectioning Oocytes and Embryos

1. Fix samples accordingly (*see* **Introduction** and **Note 1**) in 4-mL glass vials.
2. Do two changes of 100% ethanol at room temperature for 30 min each.
3. Do two changes of 100% ethanol at 39°C for 30 min each.
4. Change 100% ethanol to a 50% embedding wax [poly(ethylene glycol) distearate with 1% cetyl alcohol]/50% ethanol mix and let stand at 39°C for 90 min.
5. Change to 100% embedding wax at 39°C and let stand for 1 h.
6. Do two more changes of 100% embedding wax at 39°C for 1 h each.
7. Place samples in embedding trays with fresh wax and allow to harden.
8. Once the wax has hardened trim excess wax away with a razor blade and mount specimens, in the desired orientation for sectioning, onto small wooden blocks (*see* **Note 7**).
9. Cut 20–30 μm sections in ribbons with a microtome.
10. Float ribbons in a small puddle of distilled water on gelatin-coated slides (*see* **Note 2**). Place slides on a 29°C hot plate for 10 min. Ribbons will expand in water. Wick away remaining water.
11. Once slides have air dried place them in a desiccator overnight. This will affix sections tightly to the slides so they are not lost during staining.

3.1.2. Staining Thick Sections

1. Dewax sections using an acetone series. Slides are sequentially submerged in 100%, 90%, 70%, and 50% acetone (v/v with distilled water) for 5 min each.
2. Wash sections in 1× PBS + 1% horse serum for 15 min.
3. Block slides for 30 min to an hour in blocking buffer (10% horse or goat serum, 4% BSA in 1× PBS).
4. Incubate sections in primary antibody (diluted to the appropriate concentration in blocking buffer) in a wet box (*see* **Note 8**). The typical volume of antibody used is a puddle of about 50–100 μL per slide depending on how many sections are on the slide. Antibody solutions cannot be allowed to dry on the sections. Incubations can be at room temperature for 1–2 h or at 4°C overnight.
5. Wash sections in 1× PBS + 1% horse serum 3× 15 min each.
6. Incubate sections in fluorescently conjugated secondary antibody (diluted 1:50 in blocking buffer) for 1 h at room temperature, again in a wet box.
7. Wash sections in 1× PBS + 1% horse serum 3× 15 min each.
8. Yolk autofluorescence (*see* **Note 3**) of sections can be blocked somewhat by immersing slides in a 1:100 dilution of 1% eriochrome black (in PBS or water) for 5–10 min. Eriochrome black can be used with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies but should NOT

be used with tetramethyl rhodomine isothiocyanate (TRITC)-conjugated antibodies.
9. Rinse the sections in distilled water and mount in aqueous mounting medium (70–90% glycerol in 1× PBS) containing antifade agents (*see* **Note 4**).
10. View the specimens on a confocal laser scanning microscope. Take Z-series through 10–30 µm of an oocyte or embryo at 1–5 µm intervals. Project the images for a three-dimensional view. The program NIH Image is useful for manipulating confocal images.

3.2. Whole-mount Immunocytochemistry

(Adapted and modified from Gard and Kropf, **ref. *4***)

1. Fix oocytes or embryos accordingly in 4-mL glass vials (*see* **Introduction** and **Note 1**).
2. Bleach samples in 10% hydrogen peroxide in ethanol or methanol for 6–48 h at room temperature if you want to remove pigmentation from oocytes or embryos ***(7)*** (*see* **Note 5**).
3. Rehydrate the samples with a series of PBS washes at room temperature. Wash for 15 min in 50% PBS/50% ethanol or methanol and then wash 2× 15 min each in 1× PBS.
4. Bisect the oocytes or embryos with a scalpel blade in a Petri dish of 1× PBS.
5. Incubate samples in 100 m*M* $NaBH_4$ made fresh (in 1× PBS only) for 4 h at room temperature or overnight at 4°C to reduce aldheydes if fixed in aldehyde fixative ***(8)*** (optional, *see* **Note 6**).
6. Wash samples in TBSN (1× Tris-buffered saline + 0.1% NP-40) 3× 30 min each at room temperature. Place vials horizontally on rocker or nutator for washes.
7. Carefully transfer samples to 2-mL glass vials using a Pasteur pipet and incubate for 16–24 h at 4°C in 150–250 µL of primary antibody diluted appropriately in TBSN + 2% BSA. Place vials vertically on rocker or nutator for gentle motion.
8. Wash samples in 500 µL of TBSN for 24–36 h at 4°C, changing the wash buffer every 8–12 h. Again use gentle agitation.
9. Incubate samples at 4°C for 16–24 h in 150–250 µL of fluorochrome-conjugated secondary antibody diluted (usually 1:50) in TBSN + 2% BSA. Incubate with gentle motion.
10. Wash samples with gentle agitation in 500 µL TBSN for 24–36 h at 4°C, changing wash buffer every 8–12 h.
11. Dehydrate the samples. Remove wash buffer and add 1 mL of 50% methanol (in 1× TBS or PBS) at room temperature for 15 min. Then proceed with three 30-min rinses in 100% methanol at room temperature. Samples can be stored in methanol either at –20°C or at room temperature in a dark box.
12. Mount samples in Murray's Clear (benzyl benzoate/benzyl alcohol)(1:2 v/v) (*see* **Introduction** and **Note 9**). Carefully remove three to five bisected embryos with a Pasteur pipet and place them in a 0.5-mm cavity slide. Manipulate and

maneuver embryos with a fine forceps or hairloop. Dab methanol away and allow the rest to evaporate. Fill cavity with Murray's Clear and let sit briefly as floating embryos will eventually sink. Carefully add a coverslip and then remove excess Murray's Clear by wicking it away with a paper towel. Seal edges of coverslip by applying nail polish. Slides can be stored in the dark at room temperature or 4°C.

13. View the specimens on a confocal laser scanning microscope. Take Z-series through 10–50 µm of an oocyte or embryo at 1–5 µm intervals. Project images for a three-dimensional view. The program NIH Image is useful for manipulating confocal images.

4. Notes

1. As discussed in the introduction it is important to try a range of different fixatives. A number of fixatives have proven successful for immunostaining in *Xenopus* oocytes and embryos. All fixatives should be made fresh for each use. Following is a list of these fixatives:

   a. 100% MeOH
   b. 2% Trichloroacetic acid (in distilled water)
   c. MEMFA: 0.1 *M* MOPS (pH 7.4), 2 m*M* EGTA, 1 m*M* $MgSO_4$, and 3.7% formaldehyde
   d. FG fix: 80 m*M* K PIPES (pH 6.8), 1 m*M* $MgCl_2$, 5 m*M* EGTA, 0.2% Triton X-100, 3.7% formaldehyde, 0.25% glutaraldehyde ***(4)***
   e. 4% paraformaldehyde, 0.1% glutaraldehyde, 100 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* HEPES, 150 m*M* sucrose, and 0.1% Triton X-100 (pH 7.6) ***(9)***

2. Coated slides for thick sections need to be prepared in advance. Degrease slides by submerging in a carefully made solution of 10% potassium dichromate/concentrated sulfuric acid (1:10) for at least 24 h. Wash slides well in distilled water. Rinse slides in 95% ethanol and allow to completely air dry. Next place slides in section adhesive for 10 min and then remove, drip dry briefly, and place in a desiccator for overnight drying. Prepare section adhesive as follows. Dissolve 7.5 g of gelatin powder in 150 mL of water with heating. Dissolve 0.5 g of chromic potassium sulfate in 200 mL of water. Add 35 mL acetic acid and 145 mL of 100% ethanol to chromic potassium sulfate. Lastly, while stirring add gelatin mix.
3. *Xenopus* oocytes and cells of embryos contain many yolk platelets that autofluoresce under many commonly used excitation wavelengths. Under epifluorescent microscopy yolk autofluorescence is greater in thick sections than in whole-mounts. Under confocal microscopy, however, yolk autofluorescence becomes less apparent in early-stage embryos.
4. Antifade agents such as DABCO ***(10)***, *N*-propyl gallate ***(11)***, and *p*-phenylenediamine ***(10)*** can be added to aqueous mounting mediums. Murray's Clear itself seems to be sufficient at keeping samples from fading too rapidly. Most photobleaching occurs during confocal image collection, so care should be taken to avoid excessive laser exposure.

5. If pigmentation affects immunofluorescence, oocytes and embryos can be bleached in a solution of 10% hydrogen peroxide in ethanol for 6–48 h prior to antibody incubation *(7)*. The longer immersion times appear to make embryos more brittle.
6. Using glutaraldehyde as a fixative can often cause autofluorescence. In such cases embryos can be soaked in 100 m*M* sodium borohydride ($NaBH_4$) in PBS to reduce the unreacted aldehydes *(8)*. Care should be taken as $NaBH_4$ is very reactive with water and creates much effervescence. This treatment however, has not always been effective in our laboratory and is most often left out.
7. Sectioning wax-embedded specimens can be a difficult task if the of the room becomes warm, as specimens tend to melt. To prevent this, once samples are hardened and mounted onto cutting blocks they can be placed at 4°C for approximately an hour and removed just before cutting. Sectioning can be performed with a bucket of dry ice near by or with small chunks of dry ice placed carefully on the edges of the blade.
8. Wet boxes can be created by putting damp towels in the bottom of Tupperware containers. Slides are then placed on some sort of support system. For processing many slides we have converted commercial lucite desiccator boxes into wet boxes simply by placing wet towels in the bottom.
9. Murray's Clear is a skin irritant, so gloves should be worn and caution should be used when handling it. Inhalation should also be minimized. The solution is also detrimental for microscope lenses, so slides must be sealed properly.

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