Protocol

Whole-Mount Fluorescence Immunocytochemistry on *Xenopus* Embryos

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INTRODUCTION

Immunocytochemistry (ICC) is widely exploited in studying mammalian systems, but is underutilized among *Xenopus* developmental biologists. This stems, in part, from the relatively small number of *Xenopus* antibodies available for use in research. Common misconceptions about ICC in *Xenopus* embryos also prevail, discouraging researchers from trying the procedure. However, ICC with *Xenopus* is simple and effective. This article describes methods for whole-mount ICC in *Xenopus* embryos. Also included are simple procedures to quench autofluorescence of *Xenopus* and to remove surface pigment from embryos which may interfere with fluorescence imaging. The methods described here are useful for detecting tissue-specific probes (e.g., 12/101 to detect somites). They are also effective for imaging the cytoskeleton (e.g., α-tubulin to detect microtubules) or localizing specific proteins at the subcellular level (e.g., ZO-1 to detect tight junctions). In addition, combining ICC with in situ hybridization is simple and highly effective.

RELATED INFORMATION

Many mammalian antibodies work very effectively for *Xenopus* ICC (see Table 1). The choice of fixative is critical. Some antibodies do not work on formaldehyde-fixed embryos, but work very well on methanol-fixed embryos.

The methods described in this article have been optimized in our laboratory, and are based upon protocols described previously (Becker and Gard 2006; Robinson and Guille 1999; Sive et al. 2000). Methods for imaging embryos after ICC are described in Davidson and Wallingford (2005).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Antibodies, primary and secondary
<**R**><**!**>Autofluorescence reducing agent (optional; see Step 7)
<**!**>Hydrogen peroxide (H_2O_2) (30%, v/v) (optional; see Step 4)
<**R**><**!**>MEMFA
<**R**><**!**>*Dent's fixative can be used as an alternative, if an aldehyde-free fixative is needed (see Step 1).*
<**!**>Methanol (100%)

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<R><!>Murray's clear (for imaging below the superficial cell layer; see Step 18.iii)
<R>PTW buffer (for MEMFA fixation; see Step 1.ii)
<R>TBS for XICC
<R><!>TBST for XICC
<R><!>Whole-mount block solution (WMBS)
Xenopus embryos at desired stage

Equipment

Agarose, very thin layer (optional; see Steps 18.i, 18.ii)
Combs, plastic (optional; see Step 18.ii)
Confocal microscope, inverted (for cell-level imaging; see Step 18.ii)
Coverslips (for imaging below the superficial cell layer; see Step 18.iii)
Culture dishes with cover glass bottoms (for cell-level imaging; see Step 18.ii)
Fluorescent stereoscope and digital camera (for tissue-level imaging; see Step 18.i)
Incubator preset to 20°C-23°C (if room temperature is outside of this range)
Light box (optional; see Step 4)
Microcentrifuge tubes, cut (for imaging below the superficial cell layer; see Step 18.iii)
Mixer, rotating, or nutator
Petri dishes, plastic
Vials with caps for incubating embryos
Wax, melted (for imaging below the superficial cell layer; see Step 18.iii)

METHOD

Fixing Embryos

Embryos are fixed in either MEMFA, which contains formaldehyde, or Dent's fixative, an excellent aldehyde-free fixative. Many epitopes that cannot be detected after fixation in MEMFA are easily detected in Dent's-fixed embryos.

1. Fix embryos using one of the following methods:

To fix embryos in MEMFA

i. Incubate the embryos in 1X MEMFA either for 2 h on a rotating mixer at room temperature or overnight on a nutator at 4°C.

ii. Remove the MEMFA and wash the embryos with three 5-min washes in PTW buffer.

iii. Perform repeated 5-min washes of the embryos in 100% methanol until they are fully dehydrated. Complete dehydration is assessed by the absence of Schlieren lines (the lines

Table 1. Some mammalian antibodies that are effective for whole-mount immunocytochemistry in *Xenopus* embryos

Antibody	Supplier and catalog number	Dilution
Anti-γ tubulin	Abcam ab11321	1:500
Anti-γ tubulin	Abcam ab27076	1:500
Centrin1	Abcam ab11257	1:500
Sec8	Abcam ab13254	1:100
α tubulin	Sigma T 9026	1:300
α tubulin	Abcam ab4074	1:300
Spectrin	Sigma S 1515	1:200
ZO-1	Invitrogen (Zymed) 61-7300	1:50
PRC1	Biolegend 603001	1:50
Laminin receptor	Abcam ab2508	1:300
Phospho-Smad	Cell Signaling Technology T 9511	1:100

observed at the interface of two mixed liquids). When Schlieren lines are no longer seen, wash the embryos twice more for 5 min each in methanol.

To fix embryos in Dent's fixative

Dent's-fixed embryos are far more fragile than MEMFA-fixed embryos, because no aldehyde cross-linking is involved. Thus, for Dent's-fixed embryos, perform all subsequent steps on a nutator and never on a rotating mixer.

iv. Incubate the embryos in Dent's fixative overnight on a nutator at 4°C.

v. Remove the Dent's fixative and wash the embryos with 100% methanol.

vi. Perform repeated 5-min washes of the embryos in 100% methanol until they are fully dehydrated. Complete dehydration is assessed by the absence of Schlieren lines. When Schlieren lines are no longer seen, wash the embryos twice more for 5 min each in methanol.

Dehydrating Embryos

2. Store the embryos overnight at –20°C in methanol.

   If time is short, embryos can be stored for as little as 4 h.

3. If surface pigments need to be removed, follow Steps 4-6 and then go to Step 9. If autofluorescence is an issue, use Steps 4-8. If neither is a problem, go to Step 9.

Bleaching to Remove Pigment

These steps remove surface pigments within the embryo that can interfere with fluorescence imaging. Alternatively, albino embryos can be used.

4. Prepare 10% H_2O_2/67% methanol by combining 1 mL of 30% H_2O_2 and 2 mL of 100% methanol. Bleach pigmented embryos in this solution for 3 h in a light box.

5. Prepare 50% methanol/50% TBS for XICC and 25% methanol/75% TBS for XICC. Rehydrate the embryos slowly in consecutive 10-min (minimum) washes of:

   i. 50% methanol/50% TBS for XICC

   ii. 25% methanol/75% TBS for XICC

   iii. 100% TBST for XICC

6. Proceed to Step 7 to reduce autofluorescence. Otherwise, go to Step 10.

Reduction of Autofluorescence

Use Steps 7 and 8 if subcellular imaging is desired or any time that autofluorescence of yolk platelets is a problem.

7. Incubate the embryos in autofluorescence reducing agent for either 4 h at 20°C-23°C or overnight at 4°C. (As described in the recipe, the composition of the autofluorescence reducing agent varies with the incubation conditions.)

   Overnight is better, but do not incubate longer than that! Use caps with holes in them so pressure does not build up within the vials during the incubation. Do not lay the vials on their side—keep them upright. Do not use a shaker during this step!

8. Wash the embryos five times in TBST for XICC for 10 min each at room temperature. Go to Step 11.

Blocking the Embryos and Incubation with Antibody

9. Use rehydration solutions prepared as in Step 5 to rehydrate the embryos slowly in consecutive 10-min (minimum) washes of:

   i. 50% methanol/50% TBS for XICC

   ii. 25% methanol/75% TBS for XICC

   iii. 100% TBST for XICC

10. Wash the embryos two or three times in TBST for XICC for 5 min each.

11. Block embryos in 300-500 µL of WMBS for 30-60 min at room temperature on a nutator.
This step is not necessary for staining epidermal cilia.
12. Remove the WMBS and replace it with 300 µL of WMBS containing primary antibody.
If the volume of the WMBS is already at 300 µL, add the primary antibody directly to the WMBS.
13. Incubate the embryos with the antibody overnight at 4°C on a nutator.
14. Remove, but save, the primary antibody solution (it can be used four to five times).
15. Wash the embryos with five 1-h rinses in TBST for XICC.
16. Repeat Steps 11-13 using appropriate secondary antibody instead of primary antibody.
17. Remove the secondary antibody and wash the embryos with five 1-h rinses in TBST for XICC.
18. Image the embryos. For imaging protocol details, see Davidson and Wallingford (2005).

Tissue-level imaging

i. Use a fluorescent stereoscope and a digital camera. Place the embryos in plastic Petri dishes or on a very thin layer of agarose. (It must be a very thin layer, because agarose fluoresces!).

Cell-level imaging

ii. Use an inverted confocal microscope. Place embryos in culture dishes with cover glass bottoms. In some cases, it is helpful to add a thin layer of agarose to the cover dishes and place the embryos in wells made in the agarose with plastic combs.

Imaging below the superficial cell layer

iii. Dehydrate the embryos completely in methanol and clear them with Murray's clear. Make imaging chambers by affixing the barrel of a cut microcentrifuge tube to a coverslip using melted wax.

19. Embryos can be stored for several days in TBST for XICC. If they need to be kept longer, dehydrate them in methanol and store them at –20°C. When the embryos are needed again, slowly rehydrate them as in Step 9.

DISCUSSION

We routinely perform in situ hybridization according to Harland (1991) or use the shorter method described in the *CSH Protocols* article **A Rapid Protocol for Whole-Mount In Situ Hybridization on *Xenopus* Embryos** (Monsoro-Burq 2007). After staining for in situ hybridization with either BM Purple or NBT/BCIP, embryos are fixed in MEMFA and processed using the protocol described here. Care should be taken to handle the embryos gently, because they become very fragile after so much processing. A new method for confocal microscopy of embryos stained with NBT/BCIP or BM Purple was recently reported (Trinh et al. 2007). We have found that method to be very effective for visualizing in situs combined with ICC.

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