CHAPTER 12

Immunofluorescence Methods for *Caenorhabditis elegans*

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INTRODUCTION

The use of antibodies to visualize the distribution and subcellular localization of gene products powerfully complements genetic and molecular analysis of gene function in *Caenorhabditis elegans*. The challenge to immunolabeling *C. elegans* is finding the fixation and permeabilization methods that effectively make antigens accessible without destroying the tissue morphology or the antigen. Embryos are surrounded by a chitinous eggshell and larvae and adults are surrounded by a collagenous cuticle, each of which must be permeabilized to allow penetration of antibodies. In addition, antigens and antibodies are sensitive to different fixing and permeabilizing conditions. For example, some antibodies do not work well on paraformaldehyde-fixed samples, and others are sensitive to incubation in acetone. There are many protocols used in the *C. elegans* field; additional protocols are summarized in Miller and Shakes (1994) and on the *C. elegans* World Wide Web page (http://elegans.swmed.edu/).
Preparation and Immunolabeling of *C. elegans*

The following protocols describe the fixation of embryos, larvae, or adults for immunolabeling with antibodies.

**FIXATION OF ANIMALS AND TISSUE**

The organism may be fixed using either a whole-mount freeze-cracking method or tissue extrusion.

**Whole-Mount Freeze-cracking Method**

This method (Strome and Wood 1982; Albertson 1984; Bowerman et al. 1993) is a good starting point; it is easy and it works well with most antibodies and with embryos, larvae, and adults.

1. Assemble the following items before starting the procedure:

   - 25 gauge syringe needle
   - M9 buffer

   **M9 Buffer (1 liter)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>6.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

   Bring to 1 liter with dH₂O

   **CAUTION:** Na₂HPO₄; NH₄Cl (see Appendix 2 for Caution)

   - Formaldehyde, if used
   - TBSB

   **TBSB (enough for 1 or 2 staining experiments)**

   Add 9 ml of 1X TBS (Tris-buffered saline) to 1 ml of 5% BSA (bovine serum albumin).

   - Coplin jars containing methanol and acetone precooled on dry ice (~10 min)
   - Metal plate precooled on dry ice (~10 min)
   - Subbed slides
   - Coverslips

2. Prepare subbed slides:

   a. Prepare subbing solution:

      Bring 200 ml of dH₂O to 60°C.
      Add 0.4 g of gelatin and cool to 40°C.
      Add 0.04 g of Chrome Alum.
Add **sodium azide** to 1 mm.
Add polylysine (molecular weight [m.w.] >300,000; Sigma F 1524) to 1 mg/ml.

**CAUTION:** Sodium azide (see Appendix 2 for Caution)

b Put the subbing solution in a Coplin jar and store it at 4°C.

c Soak clean slides in subbing solution for 5 minutes to 1 hour, air-dry, and store at room temperature.

*Subbed slides can be used for weeks. Several batches of slides can be subbed in the same subbing solution. When slides become less sticky, it is time to make a new solution.*

3 Place animals into 6 µl of M9 on a subbed slide. Cut the animals open with a 25-gauge syringe needle if early embryos or extruded germ lines or intestines are to be stained.

*Use more than ten adults or more than 40 larvae; some animals or tissues will be lost from the slide during the staining procedure.*

4 Add 2 µl of 5% formaldehyde.

### 5% Formaldehyde (5 ml)

Add 0.25 g of **paraformaldehyde** to 4.2 ml dH₂O.
Add 2 µl of 4 N **NaOH**.
Heat at 65°C until dissolved.
Add 0.5 ml of 10X PBS, bring volume to 5 ml with dH₂O.
Filter and store at 4°C for no more than one week.

**CAUTION:** Paraformaldehyde; NaOH (see Appendix 2 for Caution)

*Concentrations of between 1% and 5% formaldehyde are commonly used (e.g., Bowerman et al. 1993; Evans et al. 1994; Lin et al. 1995); adjust concentration for ideal staining.*

*Formaldehyde can be omitted if it interferes with antibody binding.*

*Formaldehyde fixation improves morphology.*

5 Set an 18 x 18-mm #1 coverslip carefully on top of the animals. Use a needle to apply gentle pressure several times over each animal or region of the slide. The animals will flatten; usually a few burst. This procedure aids in opening the eggshell or cuticle. Alternatively, a Kimwipe can be used to wick excess liquid from under the coverslip until the worms flatten.

6 If formaldehyde was added, let the sample incubate 30 seconds to 30 minutes at room temperature in a humidified chamber (e.g., Bowerman et al. 1993; Evans et al. 1994; Lin et al. 1995); adjust time for ideal staining.

*A humidified chamber can be made from a plastic petri dish (or other container) with a wet paper towel taped to the lid.*

7 Put the slide on a metal plate on top of dry ice for at least 10 minutes.

*We use plates 1/4" thick. The plates are used to make a cold, flat surface for the animals to sit on.*

8 Pop the coverslip off with a razor blade and immerse the slide immediately in 100% cold **methanol** for 5 minutes, followed by 100% cold **acetone** for 5 minutes.

**CAUTION:** Methanol; acetone (see Appendix 2 for Caution)

*For some antigens, it is better to omit the acetone incubation.*

*For some antigens, incubation in cold DMF (N,N-dimethylformamide) works better than methanol or acetone (Lin et al. 1998).*
9 Air-dry the slide for 5 minutes. This enhances the adhesion of the animals to the slide. Rehydrate the sample through a series of increasingly aqueous solutions. Either methanol (90%, 70%, 50%, followed by 1x TBS; Lin et al. 1995), ethanol (2 minutes each in 95%, 70%, 50%, and 30%, followed by 1x TBS; Miller and Shakes 1994), or acetone (Goldstein and Hird 1996) series have been used.

10 Gently drop 200 μl of TBS containing 0.5% BSA (TBSB) onto the animals and incubate at room temperature for 30 minutes.

11 Follow the antibody incubation procedure (see below).

**Notes**

- Larvae and adults can either be picked from plates or washed off with M9. Young embryos (1–50 cells) are easily obtained by cutting open gravid hermaphrodites. Older embryos can be obtained by adding M9 to a plate, washing off the adults and larvae, then scraping the remaining embryos off with a pasteur pipette into additional M9. The worms or embryos are then pelleted by spinning for 1–2 minutes at 1000 rpm in a microfuge. To remove *Escherichia coli*, more M9 is added, and the worms are pelleted again. Then 6–8 μl of concentrated worms can be dropped onto a slide.

- When staining larvae, it helps to stage the animals so that they are similar in size. This way the amount of pressure can be adjusted for the size of the worms being fixed. For example, if there are lots of large adults (or larvae), it is difficult to permeabilize L1s without completely squashing the adults. It gets increasingly more difficult to effectively permeabilize the worms as they get older.

- For tissues that can be extruded from the cuticle, such as germ lines and intestines, the morphology is generally better using a non-freeze-crack method (see extrusion method).

- Another commonly used procedure for whole mounts is the reduction/oxidation method of Finney and Ruvkun (1990). This method is described in detail in Miller and Shakes (1995).

**Tissue Extrusion Method**

Using this method (Crittenden et al. 1994), gonads and intestines, which are extruded from the carcass, are well fixed and permeabilized (Fig. 12.1A,B). Tissues remaining in the carcass are not usually stained well.

1 Assemble the following items before starting the procedure:

- 0.25 mM levamisole (Sigma)

<table>
<thead>
<tr>
<th>0.25 M Levamisole (5 ml)</th>
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</thead>
<tbody>
<tr>
<td>Dissolve 0.3 g of levamisole in 5 ml of M9. Aliquot and store at −20°C. For 0.25 M levamisole, add 1 μl of 0.25 M levamisole to 1 ml of M9. Store at room temperature.</td>
</tr>
</tbody>
</table>

- 25 gauge syringe needle
- 1.0% formaldehyde
- TBSB
FIGURE 12.1

Gonads from wild-type *C. elegans* hermaphrodites were fixed according to the tissue extrusion method. Primary antibodies were rat anti-GLP-1 (Crittenden et al. 1994) and mouse anti-DNA (Chemicon, MAB030). Secondary antibodies were Cy3-conjugated donkey anti-rat and FITC-conjugated donkey anti-mouse (Jackson Immunoresearch). Images were obtained on a Bio-Rad MRC 1024 laser scanning confocal microscope. (A,B) Well-fixed gonad. The transmembrane receptor, GLP-1, is membrane-associated in a crisp honeycomb pattern. DNA staining is crisp, and discrete structures within the nucleus are visible. (C,D) Poorly fixed gonad. GLP-1 is punctate and diffuse. In addition, there is a faint red haze in the nuclei. DNA staining is bright, but nuclear morphology is poor; the nuclei are fuzzy and discrete structures are not discernible. The poor morphology was due to a contaminant in the primary antibody solution. (Photo provided by S. Crittenden, University of Wisconsin.)

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**TBSB (enough for 1 or 2 staining experiments)**

Add 9 ml of 1x TBS to 1 ml of 5% BSA.

TBSBTx (TBSB with 0.1% Triton X-100)
Subbed slides

2 Put five to ten adult hermaphrodites into 5 μl of M9 containing 0.25 mM levamisole on a subbed slide. Using a 25-gauge syringe needle, cut off the heads or tails of the animals, allowing the gonad and intestine to extrude from the animal.

*Levamisole causes the animals to contract, which results in their germ lines and intestines being extruded efficiently.*

3 Gently drop 100 μl of 1.0% formaldehyde onto the cut animals. Incubate at room temperature for 10 minutes in a humidified chamber.

**CAUTION:** Formaldehyde (see Appendix 2 for Caution)

4 Remove the formaldehyde and add 50 μl of TBSB containing 0.1% Triton X-100 (TBSBTx) at room temperature for 5 minutes.

5 Remove the TBSBTx and wash two times with 200 μl of TBSB.

6 Incubate samples in 200 μl of TBSB at room temperature for ~30 minutes.

7 Follow the antibody incubation procedure.
Notes

- This fix works well for at least some membrane proteins (Crittenden et al. 1994; Henderson et al. 1994), but not for the cytoskeletal proteins actin and tubulin.

- For cytoskeleton, try fixing first in 100% methanol at room temperature for 5 minutes followed by 1% formaldehyde at room temperature for 25 minutes (Crittenden et al. 1994).

- Other protocols have been used for fixing extruded tissues (Strome 1986; Francis et al. 1995) and/or the cytoskeleton (Strome 1986; Waddle et al. 1994; Francis et al. 1995).

ANTIBODY INCUBATION PROCEDURE

Worms fixed according to the preceding protocols are incubated overnight with primary antibody, subsequently exposed to secondary antibody, and mounted for viewing.

1 Incubate fixed worms with primary antibodies overnight at 4°C or for several hours at room temperature in a humidified chamber. Use 30–50 µl of antibody solution per slide.

2 Wash by gently covering the worms with 200 µl of TBSB. Wash three times for 15 minutes each at room temperature. Alternatively, the slides can be immersed in a Coplin jar if the worms are well attached to the slide.

3 Dilute secondary antibodies to the recommended concentration in TBSB. Use 100 µl per slide and incubate at room temperature for 1–2 hours.

We use Jackson Immunoresearch's purified IgG coupled to Cy3, Cy5, FITC (fluorescein isothiocyanate), or rhodamine.

To reduce nonspecific background, add ~1 mg of worm acetone powder/200 µl of secondary antibody solution.

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### Acetone Powder (modified from Harlow and Lane 1988)

Homogenize worms in Dounce homogenizer; use about 1 g of worms/ml of M9.
Set on ice for 5 minutes.
Add 4 ml of ~20°C acetone/ml of worm suspension. Mix vigorously.
Set on ice for 30 minutes with occasional vigorous mixing.
Spin at 10,000g for 10 minutes.
Resuspend pellet with fresh ~20°C acetone.
Mix vigorously on ice for 10 minutes.
Spin at 10,000g for 10 minutes.
Spread pellet on clean filter paper and allow to dry at room temperature. When dry, break up chunks in a mortar and pestle, then transfer powder to a microfuge tube and store at 4°C.

**CAUTION:** Acetone (see Appendix 2 for Caution)

Incubate the secondary antibody/worm acetone powder mix at 4°C for 15 minutes to 1 hour. Then centrifuge at 10,000 rpm for 5 minutes in a microfuge to pellet the acetone powder. Use the supernatant as the secondary antibody solution.

4 Wash worms as in step 2. If desired, add the DNA stain, DAPI (4',6-diamidino-2-phenyldole), to 0.5 µg/ml in the final wash.

5 After removing the last wash, wick off excess moisture from the slide by touching the edges with a Kimwipe. Add 8 µl of mounting medium (see Chapter 4), put an 18 x 18-mm #1 coverslip over the worms, and seal with nail polish.
Notes

- Worms generally do not stick well to slides, so start with plenty of animals and be gentle when doing washes.
- If background is high, determine whether it is due to the primary or secondary antibody. Try diluting the antibodies further, affinity-purify the primary antibody, and preabsorb the primary antibody with worm or bacterial acetone powder or with fixed worms; null mutants should be used so that the specific antibody will not be depleted.
- Do not let the worms dry after they have been fixed and rehydrated; this tends to give a nonspecific haze to the nuclei and cytoplasm.
- If the morphology looks poor, try to fix the worms more quickly. Alternatively, contaminated solutions can cause poor morphology. DAPI-stained DNA should look well defined and crisp; if it does not, be suspicious (e.g., Fig. 12.1 C, D).
- Common background problems include intestine autofluorescence on the DAPI and fluorescein channels, causing a dim nuclear stain.
- Using different fixatives or making small changes in concentration of fixative or time of fixation can make a big difference in the quality of staining.

CONTROLS

- Use an antibody that is known to work to test for morphological preservation, permeability, and fixation. Some useful control antibodies are anti-DNA monoclonal mAb 030 (Chemicon, MAB030), anti-actin clone C4 (ICN Biochemicals, 69-100-1) (Strome 1986; Evans et al. 1994), anti-β-tubulin (Amersham, N357) (Crittenden et al. 1994; Evans et al. 1994; Waddle et al. 1994).
- Stain a null mutant and look for loss of staining.
- Compete for staining with proteins that contain the antigen used to raise the antibodies.
- If a null mutant is not available, it is possible to abolish the antigen in embryos from animals that have been injected with antisense RNA (Guo and Kemphues 1995).

REFERENCES

2913-2924.

