Protocol

Immunostaining for Markers of Apoptosis in the *Caenorhabditis elegans* Germline

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The transparency of *Caenorhabditis elegans* makes it an ideal organism for visualizing proteins by immunofluorescence microscopy; however, the tough cuticle of worms and the egg shell surrounding embryos pose challenges in achieving effective fixation so that antibodies can diffuse into cells. In this protocol, we describe immunostaining of apoptosis-related proteins in the *C. elegans* adult germline using fluorescent reagents. Protein localization and abundance can be determined in various mutant backgrounds and under a variety of conditions, such as exposure to genotoxic stress. The number of antibodies specific to *C. elegans* proteins is quite limited compared with other organisms, but there is a growing list of immunological reagents directed against proteins in other organisms that cross-react with the homologous *C. elegans* proteins.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

4′,6-Diamidino-2-phenylindole (DAPI) (optional; see Step 18)

DNA can be stained with DAPI to assess nuclear morphology and/or quantify nuclei in the germline. Prepare a solution of 1 µg/mL DAPI in PBST.

Acetone (chilled to −20°C)

Antibody (primary)

Antibody (secondary, fluorescently labeled)

Blocking solution

Prepare a blocking solution of 0.1% BSA in PBST.

Bovine serum albumin (BSA)

C. elegans strain of interest, at the L4 stage

Always use strains with a null mutation in the gene corresponding to the protein of interest as negative controls to ensure antibody specificity. If mutants are not available, RNA interference (RNAi) can be used to knockdown the

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gene; see Protocol: ***Induction of Germline Apoptosis in* C. elegans** *(Lant and Derry 2014). The method described here can also be used to determine if RNAi clones are working as expected.*

Image-iT FX signal enhancer (Life Technologies)
Methanol (chilled to −20°C)
Paraformaldehyde solution (4%) <R>

On the day of staining, prepare a solution of 2% paraformaldehyde by diluting the 4% solution in an equal volume of 1× PBS <R>.

PBST

Prepare a PBST solution of 0.1% Tween-20 in 1× PBS.

Phosphate-buffered saline (PBS) (1×) (pH 7.4) <R>
Plates containing solid nematode growth medium (NGM) <R>, seeded with OP50 or RNAi bacteria
ProLong Gold antifade reagent (Invitrogen P36930)
Tetramisole (20 mM)
Triton X-100

Prepare a solution of 1% Triton X-100 in 1× PBS <R>.

Unique reagents required for preparing acetone blocking powder (optional; see Steps 22–31):

- Acetone (chilled to −20°C)
- *C. elegans*

  We recommend using at least six large (10-cm diameter) plates worth of young adult worms for preparation of acetone blocking powder.

- NaCl (0.1 M, chilled)
- Sucrose (60% in 0.1 M NaCl)

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Equipment

Centrifuge
Compound microscope with differential interference contrast (DIC) and fluorescence optics
Coverslips
Dry ice
Forceps
Glass transfer pipettes

Glass pipettes are recommended because worms are prone to stick to plastic tips.

Humidified chamber at room temperature
Imaging software
Metal heating block
Nail polish
Needles (25-gauge)
Razor blade
Sealing wax
Slides coated with poly-L-lysine
Tubes (2-mL)
Unique equipment required for preparing acetone blocking powder (optional; see Steps 22–31):

- Incubator at 37°C
- Liquid nitrogen
- Microcentrifuge tubes
- Mortar and pestle
- Petri dishes (sterile plastic)
- Rotating platform
- Tubes (15-mL conical)

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METHOD

Steps 1–21 describe the standard immunostaining procedure. If you notice high background signal after staining, we recommend repeating the procedure and blocking the specimens before staining using an acetone worm powder, prepared as in Steps 22–31.

Immunostaining Dissected Gonads

Gonad dissection (Step 4) can be a difficult skill to master. It requires a steady hand and should be practiced before excising the gonads of target worms for fixation and staining. Finding the ideal pressure for fixing excised gonads to slides (Step 7) also may take some practice.

If you wish to compare staining between different genotypes or assay conditions, all samples should be prepared on the same day with the same solutions and antibody dilutions. Small variations in antibody binding can have large effects on signal intensity. In addition, for all antibody treatments, staining, and subsequent washes, it is crucial that the durations are equivalent between controls and replicates. Variation in incubation times can affect signal strength.

1. One day before staining, select at least 50 L4-stage worms per condition and incubate them on an NGM plate containing bacteria overnight at room temperature.
2. On the following day, wash the target worms off the plate with 1 mL of 1× PBS. Transfer the worms, in PBS, into a 2-mL tube and centrifuge at 2000 rpm for 1 min. Decant the supernatant and discard.

   A worm pellet of ∼70 µL should remain.
3. Using a glass transfer pipette, transfer the pellet of worms onto a coated slide. Apply a 1-µL drop of 20 mM tetramisole on top of the worms to immobilize them.
4. Isolate a single immobilized worm. Using two 25-gauge needles like scissors, make cuts below the pharynx and above the tip of the tail to remove the head and tail from the worm so that the gonad extrudes from the body.

   It is important to work quickly, as gonads will not pop out of the decapitated bodies after ∼10 min in solution.
5. After ∼50 gonads have been extruded, apply 30 µL of 2% paraformaldehyde solution to the worms. Incubate the slide at room temperature for 10 min.

   If gonads detach from slide at this step, see Troubleshooting.
6. Remove the excess fluid carefully with a pipette or capillary and place a coverslip diagonally over the sample, such that the corners of the coverslip hang off the edge of the slide. Press down gently on the coverslip with the back of a pair of forceps.

   Be sure to leave enough of the coverslip overhanging such that it can be used for leverage, otherwise the glass will break during coverslip removal in Step 9. Be careful not to exert too much pressure and disintegrate the germlines.
7. Place the slide on a cold metal block supported on a bed of dry ice for at least 15 min until the dissected worms and germlines freeze to the slide.
8. Repeat Steps 2–7 for any additional plates of worms.
9. Use a razor blade to carefully lever the coverslips off the slides and immediately immerse the slides in ice-cold 1:1 methanol:acetone at −20°C for 1 min.
10. Remove each slide from the solution and allow it to dry.
11. To permeabilize the samples, wash the slides with 1% Triton X-100 for 10 min at room temperature.
12. Apply 200 µL of PBST to each slide, cover with a piece of Parafilm to prevent evaporation, and incubate for 10 min in a humidified chamber. Remove the excess fluid. Repeat this step two more times.
13. Apply two drops of Image-iT FX signal enhancer to each slide and incubate for 20 min in a humidified chamber.

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14. Remove the excess fluid and add 200 µL of blocking solution to each slide. Incubate for 30 min in a humidified chamber.
15. Remove the excess fluid and add 50 µL of primary antibody (dilute antibody to appropriate concentration in PBST + 1% BSA) to each slide. Cover the slides with sealing wax to prevent evaporation and incubate overnight in a humidified chamber.

    Alternatively, primary antibody can be combined with acetone blocking powder to reduce nonspecific staining. Preparation and use of acetone blocking powder is outlined in Steps 22–31.
16. On the following day, wash the slides three times with 200 µL of PBST for 10 min per wash in a humidified chamber.
17. Add 50 µL of secondary antibody (diluted to 1:500 in PBST + 1% BSA) to each slide. Incubate the slides for 1 h in a humidified chamber.
18. (Optional) Perform DAPI staining as follows.
    i. Wash the slides twice with 200 µL of PBST for 10 min per wash in a humidified chamber.
    ii. Incubate slides in DAPI solution for 10 min.
19. Following the addition of secondary antibody (and DAPI, if applicable) staining, wash the slides three times with 200 µL of PBST for 10 min per wash in a humidified chamber.
20. Mount the samples in ProLong Gold, using less than one drop to prevent coverslip movement. Apply a coverslip to each slide and incubate in the dark overnight at 4°C.
21. On the following day, seal the coverslips to the slides using a light coating of nail polish and visualize samples by fluorescence microscopy.

    Although it seems counterintuitive not to seal the slide overnight, this will allow excess PBST to evaporate which in turn improves visibility.

    Immunostaining for a given protein target or phosphorylation site can be assessed qualitatively (as the presence or absence of signal) or by quantitative analysis, which is a standard capability of most imaging software. If you are looking at staining in the nuclei, it is important that intensity values are normalized to the intensity values of the surrounding cytosol. It is also critical to use the same exposure and gain settings on the imaging software when comparing intensities between samples.

    See Troubleshooting.

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Preparing Acetone Blocking Powder

22. Grow worms to adulthood on 5 × 10 mm NGM plates then wash the worms from the plates into a 15-mL conical tube using 1× PBS. Centrifuge the worms at 1000 rpm for 2 min to pellet, and carefully remove the supernatant.

    When centrifuging worms for this procedure, you will often notice a "tornado" of worms following a spin. Resist the urge to crank up the RPMs! (Although technically worms will survive spins of 5000 rpm.) Allow the samples to settle after each spin and take extra care removing the supernatant, as the top layer of worms will not be overly compact.

    It is important to obtain a sufficient quantity of adult worms for preparation of the acetone blocking powder. We recommend predetermining the number of L1 stage worms for each mutant strain that will nearly starve out a 100-mm plate by the time they develop to adults. We also recommend growing dense lawns of OP50 bacteria to maximize the number of worms for this procedure.
23. Wash the pellet three times with cold 0.1 M NaCl. Centrifuge at 1000 rpm for 2 min and remove the supernatant after each wash.
24. Fill the tube with 5 mL of cold 0.1 M NaCl and 5 mL of 60% sucrose and mix. Centrifuge at 2000 rpm for 3 min. Use a glass transfer pipette to collect and transfer the worms to a fresh tube.
25. Resuspend the worms in 10 mL of cold dH_2O, centrifuge at 1000 rpm for 2 min and remove the supernatant. Repeat for a second wash with cold dH_2O.
26. Fix the worms with 5 mL of 4% paraformaldehyde at 4°C for 4 h on a rotating platform. Once the fixation is complete, centrifuge the worms at 1000 rpm for 2 min and remove the supernatant.

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The volume of paraformaldehyde is dependent on how many worms you are using; 5 mL corresponds to six large plates.

27. Wash the worms with 10 mL of PBST, centrifuge at 1000 rpm for 2 min and remove the supernatant.
28. Incubate the worms with 5 mL of cold acetone for 20 min. Centrifuge at 1000 rpm for 2 min and remove the supernatant.
29. Wash the worms twice with 10 mL of PBST followed by once with 10 mL of PBS. Centrifuge at 1000 rpm for 2 min and remove the supernatant after each wash.
30. Transfer the worm pellet into a mortar, cover the sample with liquid nitrogen, and grind it into a fine powder with a pestle. Place this homogenate on a sterile plastic Petri dish and dry overnight in a 37°C incubator.

    The worm homogenate powder can be stored in microcentrifuge tubes at 4°C for subsequent experiments.
31. To use the powder during immunostaining, mix the powder with just enough blocking solution to form a paste. Add the target antibody to a 1:1 ratio (i.e., 50 µL of paste to 50 µL of primary antibody) and proceed with Step 15.

TROUBLESHOOTING

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Problem (Step 5): The extracted gonad will not stick to the slide with the addition of liquids.

Solution: Once you have extracted the gonads onto the poly-L-lysine-coated slide, the subsequent application of liquids can loosen the samples from the slide. If you experience this, you may need to apply more coats of poly-L-lysine solution to the slides to ensure the samples stick. Be gentle when handling slides with immobilized germlines!

Problem (Step 21): The fluorescent signal of the target protein is too low.

Solution: There are a number of possible explanations. Most importantly, you may be seeing a significant result (i.e., a biologically relevant variation in protein expression). Alternatively, there may have been an error in antibody dilution or the antibodies may have denatured. Consider changing either the primary or secondary antibody dilution to increase signal. If you have a worm strain that overexpresses the target protein, you can use it to ensure the antibodies are working well.

Problem (Step 21): Background fluorescence is too high.

Solution: If you have not tried it already, use the acetone blocking powder. The use of Image-iT FX Signal Enhancer should also reduce the level of nonspecific fluorescence, but it may be necessary to dilute the antibodies to reduce background signal. Finally, increasing the number of washes following either primary or secondary antibody incubation will also help optimize the signal-to-background ratio.

Problem (Step 21): Germlines appear damaged.

Solution: This may be an issue with fixation. Reduction of fixation time to 5 min may eliminate germlines that appear altered/damaged in the visualization step. Alternatively, germlines can appear to have exploded if the coverslip is pressed on with too much force. Practice, practice, practice!

DISCUSSION

Immunostaining allows for in situ visualization of numerous protein features, such as subcellular localization or relative levels in different genetic backgrounds or environmental conditions. Germline immunostaining is a relatively straightforward technique but requires some practice and optimization to achieve the best results. The largest issue to consider is reproducibility of staining. A second

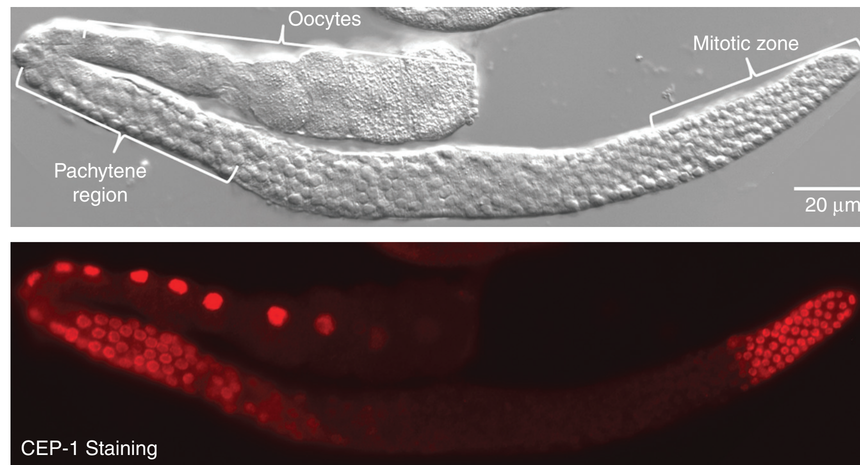

FIGURE 1. CEP-1 immunostaining of an excised germline. The *top* panel shows the white light image, clearly indicating the regions of the germline (moving *right* to *left*, from the distal [mitotic] tip through the meiotic transition zone to the bend of the pachytene region and into the developing region of the oocytes). The *bottom* panel shows CEP-1 staining, indicating strong signal in the mitotic zone, pachytene region, and oocytes. Germlines visualized at 400× magnification.

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common problem is the abundance of background signal. However, using acetone blocking powder in conjunction with an antifade reagent (i.e., ProLong Gold) should ensure that images are fairly clean.

Unfortunately, there are a limited number of antibodies available which are specific to *C. elegans* proteins, but the list is growing. Antibodies to *C. elegans* core apoptosis proteins have proven to be very useful for a number of studies in the germline and soma (Greiss et al. 2008; Ito et al. 2010; Sendoel et al. 2010; Pourkarimi et al. 2012). However, most of these reagents are not commercially available and therefore require making polite requests from the *C. elegans* researchers in whose laboratories they were generated. For the intrepid, *C. elegans* antigens can be used to develop antibodies; this process is described in the review by Shakes et al. (2012).

Despite the availability of worm-specific antibodies as a limiting factor, many antibodies to well-conserved proteins or epitopes work quite well in *C. elegans*. For example, antibodies raised to the diphosphorylated form of mammalian Erk cross-react with phosphorylated MPK-1 in the *C. elegans* germline (Rutkowski et al. 2011; Perrin et al. 2013). These antibodies are emerging as important reagents for analyzing apoptotic signaling in the germline, because the MAPK pathway promotes both physiological and stress-induced apoptosis in this tissue (Kritikou et al. 2006; Rutkowski et al. 2011; Perrin et al. 2013). Antibodies to the p53-like protein CEP-1 are also commercially available (Santa Cruz Biotechnology) (Fig. 1), and antibodies that recognize ATM consensus phosphorylation sites (pS/TQ) can be used to detect these epitopes in *C. elegans* as a readout for DNA damage checkpoint activation (Vermezovic et al. 2012).

RELATED TECHNIQUES

Immunostaining can be performed using embryos as well as whole worms (both larva and adults); see the review by Shakes et al. [2012] or refer to *WormBook* (Duerr).

Individual proteins can be isolated from worm tissue and detected by immune (western) blotting for quantification (via densitometry) of protein levels in response to mutation or stress. This procedure is described in *WormBook* (Duerr). The approach holds the caveat of requiring high protein concentrations, such that typically the whole worm (rather than a specific tissue) is used for preparation.

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RECIPES

Nematode Growth Medium (NGM)

1. For solid NGM, mix 3 g of NaCl, 2.5 g of peptone, and 20 g of agar and bring to 1 L with H_2O. (For liquid NGM, prepare without agar.)
2. Autoclave for 1 h.
3. Let cool for 1 h in a 55°C water bath.
4. Add 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M (pH 6.0) KPO_4, mixing after each addition.
5. Using an automated plate pourer (peristaltic pump), pour the medium into sterile plastic plates to set. During pouring, keep the NGM on a hot plate with stirrer to prevent the medium from solidifying.
6. Store NGM plates at 4°C until use. Seed NGM plates with bacteria the day after pouring. Warm the plates to room temperature before adding the worms. (For liquid NGM, store at room temperature for up to 1 mo. Regularly check for excess cloudiness to ensure bacterial contamination is absent.)

Paraformaldehyde Solution (4%)

1. Add 30 mg of paraformaldehyde to 1 µL of 1 M NaOH in 386 µL of H_2O. Incubate at 60°C in a heat block and vortex intermittently until the paraformaldehyde enters solution. (If this process takes longer than 30 min, an additional 1 µL of 1 M NaOH can be added.)
2. Place the heat block containing the paraformaldehyde solution on dry ice, cover, and allow the solution to cool.
3. Add 386 µL of phosphate buffer for worms.
4. Store on ice until use.

   This solution must be prepared fresh every time.

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM
If necessary, PBS may be supplemented with the following:				
$CaCl_2 \cdot 2H_2O$	0.133 g	1 mM	1.33 g	10 mM
$MgCl_2 \cdot 6H_2O$	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature.

Phosphate Buffer for Worms

Add 200 mg of KH_2PO_4 to 940 mg of Na_2HPO_4 in 25 mL of H_2O. Adjust pH to 7.2. Store at 4°C or room temperature for up to 1 mo.

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