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Confocal Methods for *Caenorhabditis elegans*

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1. 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*. Double and triple staining protocols are particularly useful for several reasons. First, colocalization of proteins either within tissues or at a subcellular level can be examined. Second, costaining with stage-specific or tissue-specific markers can define the timing and tissue specificity of antigen expression. For these types of studies it is useful to be able to collect data from multiple fluorescence wavelengths simultaneously. A confocal microscope equipped with a krypton/argon laser can simultaneously detect up to three different antigens. Using a confocal microscope it is also possible to collect a series of optical sections through a sample that allows observation of changes in distribution of the antigen in different focal planes of the tissue or cell.

In this chapter we outline procedures for fixing and staining *C. elegans* adult tissues, embryos, and larvae. Because embryos have an impermeable chitinous eggshell and larvae and adults have an impermeable collagenous cuticle, it is important to permeabilize animals without destroying morphology or antigenicity. We routinely use two different procedures depending on which tissue and which stage we want to stain. Whole mount freeze cracking ***(1–3)*** has been used for years and is a good starting point; it is easy and it works well with most antibodies and with embryos, larvae, and adults. In addition the fixation can be varied easily to suit different antigens. Another useful method is to extrude tissues, e.g., gonads and intestines, from the carcass ***(4,5)***. The extruded tissues are then easily fixed and permeabilized. Tissues remaining in the carcass usually do not stain well. This procedure is most useful for L4 larvae and

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adults, although it is possible to extrude tissues from younger larvae, too. In addition, we discuss DNA binding dyes that are useful with the confocal microscope and procedures for obtaining images from live worms expressing green fluorescent protein (GFP) reporter constructs.

2. Materials

1. Subbing solution for slides:
   a. Bring 200 mL of distilled water to 60°C.
   b. Add 0.4 g of gelatin
   c. Cool to 40°C.
   d. Add 0.04 g of chrome alum
   e. Add 1 m*M* sodium azide
   f. Add polylysine to 1 mg/mL

   Put the subbing solution in a coplin jar and store it at 4°C. Soak clean slides in subbing solution for 5 min to 1 h, 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 new solution.
2. 10× TBS (1 L): 250 m*M* Tris (pH 8.0), 1.5*M* NaCl, (30 g of Tris-base, 80.2 g NaCl). Add distilled water to 800 mL, pH to 8.0 (approx. 15 mL HCl). Bring volume to 1 L with distilled water.
3. 5% Bovine serum albumin (BSA) (makes 25 1-mL aliquots): 1.25 g BSA (FractionV), 2.5 mL of 10× TBS (pH 8.0), 22.5 mL of distilled water.

   Mix well, make 1-mL aliquots in 15-mL tubes and freeze at –20°C.
4. TBSB (enough for one or two staining experiments): Add 9 mL of 1× TBS to 1 mL of 5% BSA.
5. Acetone powder (modified from Harlow and Lane, **ref. *6***)
   a. Homogenize worms in Dounce homogenizer - use about 1g of worms/mL of M9.
   b. Set on ice for 5 min.
   c. Add 4 mL of –20 acetone/mL of worm suspension. Mix vigorously.
   d. Set on ice for 30 min with occasional vigorous mixing.
   e. Centrifuge at 10,000*g* for 10 min.
   f. Resuspend pellet with fresh –20 acetone.
   g. Mix vigorously on ice for 10 min.
   h. Centrifuge at 10,000*g* for 10 min.
   i. Spread pellet on clean filter paper and allow to dry at room temperature.
   j. When dry, break up chunks in a mortar and pestle, then transfer powder to a microfuge tube and store at 4°C.
6. M9 Buffer (1 L): KH_2PO_4 (3.0 g), Na_2HPO_4 (6.0 g), NaCl (0.5 g), NH_4Cl (1.0 g). Bring to 1 L with distilled water
7. 10× PBS (1 L): 80 g of NaCl, 2.0 g of Kcl, 14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4. Add distilled water to 800 mL (pH to 7.2), bring volume to 1 L.
8. 5% Paraformaldehyde (5 mL).
   a. Add 0.25 g of paraformaldehyde to 4.2 mL of distilled water.
   b. Add 2 µL of 4*N* NaOH.

c. Heat at 65°C until dissolved.
d. Add 0.5 mL 10× PBS, Bring volume to 5 mL with distilled water.
e. Filter.
f. Store at 4°C for no more than 1 week.

9. 0.25 *M* levamisole (5 mL): Dissolve 0.3 g of levamisole in 5 mL of M9. Aliquot and store at –20°C. For 0.25 m*M* levamisole, add 1 µL of 0.25*M* levamisole to 1 mL of M9. Store at room temperature.
10. Fluorescently labeled secondary antibodies (Jackson Immunoresearch, 872 West Baltimore Pike, P.O. Box 9, West Grove, PA 19390; 800/367-5296).
11. Propidium iodide (Molecular Probes; Catalog No. P-3566; 4849 Pitchford Ave., Eugene, OR 97402; 541/465-8300).
12. To-Pro™-3 (Molecular Probes; Catalog No. for sampler kit N-7566; 4849 Pitchford Ave., Eugene, OR 97402; 541/465-8300). Sampler kit is a good value and you can try six different dyes.
13. DAPI (4',6-diamidino-2-phenylindole) (Sigma; Catalog No. D-1388, P.O. Box 14508, St. Louis, MO. 63178; 800/325-3010).
14. Mab030 (Chemicon; Catalog No. MAB030; 28835 Single Oak Dr., Temecula, CA 92590; 800/437-7500).
15. Anti-β tubulin (Amersham; Catalog No. N357; Life Sciences Division, 2636 S. Clearbrook Dr., Arlington Heights, IL 60005; 800/323-9750).
16. Anti-phosphohistone H3 (Upstate biotechnology; Catalog No. 06-570; 199 Saranac Ave. Lake Placid, NY 12946; 800/233-3991).
17. anti-actin (monoclonal C4; ICN; Catalog No. 69-100-1; ICN Plaza, 3300 Hyland Ave., Costa Mesa, CA 92626; 714/545-0113).
18. anti-GFP (Clontech; Catalog No. 8363; 1020 East Meadow Circle, Palo Alto, CA 94303; 800/662-2566).
19. *C. elegans* website: http://eatworms.swmed.edu.

3. Methods

3.1. Fixation Methods

3.1.1. Whole-Mount Freeze Cracking Method (see **Notes 1–6***)*

1. Using a pick, 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; to try whole larvae, leave the animals intact.
2. Add 2 µL of 5% paraformaldehyde (*see* **Note 7**).
3. Set an 18 mm ×18 mm 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 egg-shell or cuticle (*see* **Note 8**).
4. Put the slide on a metal plate on top of dry ice for at least 10 min.
5. Pop the coverslip off with a razor blade and immerse the slide immediately in 100% cold methanol for 5 min followed by 100% cold acetone for 5 min (*see* **Note 7**).

6. Air dry the slide for 5 min. This helps the animals adhere to the slide better.
7. Gently drop 200 µL of TBS containing 0.5% BSA (TBSB) onto the animals and incubate for 30 min at room temperature.
8. Follow the antibody incubation procedure (**Subheading 3.2.**).

3.1.2. Tissue Extrusion Method (see **Note 9***)*

1. Using a pick, place 10–15 animals into 5 µL of M9 containing 0.25 m*M* 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 (*see* **Note 10**).
2. Gently drop 100 µL of 1.0% paraformaldehyde in PBS onto the cut animals. Incubate for 10 min at room temperature in a humidified chamber.
3. Remove the paraformaldehyde and add 50 µL of TBSB containing 0.1% Triton X100 (TBSBTx) for 5 min at room temperature.
4. Remove the TBSBTx and wash two times with 200 µL of TBSB.
5. Incubate samples in 200 µL of TBSB for approximately 30 min at room temperature.
6. Follow the antibody incubation procedure (**Subheading 3.2.**).

*3.2. Antibody Incubation Procedure (*see **Notes 11–15***)*

1. Incubate worms with 30–50 µL of primary antibody overnight at 4°C or for several hours at room temperature in a humidified chamber.
2. Wash by gently covering the worms with 200 µL of TBSB three times for 15 min each at room temperature (*see* **Note 16**).
3. Dilute secondary antibodies to the recommended concentration in TBSB. Use 100 µL per slide. If desired, add the DNA stain DAPI to 0.5 µg/mL. To reduce nonspecific background, preabsorb with worm acetone powder (*see* **Note 17**).
4. Incubate the worms with the secondary antibody mix for 1–2 h at room temperature.
5. Wash worms as in **step 2**.
6. After removing the last wash, add 8 µL of mounting medium (Vectashield, Vector Laboratories) and wipe off excess moisture with a tissue. Then put an 18 mm × 18 mm coverslip over the worms and seal with nail polish.
7. After the nail polish is dry, worms can be viewed through the confocal microscope (*see* **Note 18**).

3.3. DNA Binding Dyes

3.3.1. DAPI

DAPI staining is useful for finding worms by epifluorescence and gives the best detail if you want to look at nuclear morpholgy. DAPI images can be obtained on confocals that have UV capability.

1. Add 0.5 µg/mL of DAPI to the secondary antibody mix and proceed according to the antibody staining procedure (**Subheading 3.2.**).

3.3.2. To-Pro-3

To-Pro-3 emits in the far-red range and can be used with a krypton/argon laser. Staining with To-Pro-3 does not work as consistently or with as much detail as DAPI; however, for marking nuclei it works well (*see* **Notes 19** and **20**).

1. After the secondary antibody incubation and one wash, add a 1:5000 to 1:10,000 dilution of a 1 m*M* stock to 200 µL of TBSB per slide.
2. Incubate at room temperature for 10 min.
3. Wash three more times as with TBSB and mount the slides as described in the antibody staining procedures (**Subheading 3.2.**).

3.3.3. Propidium Iodide

Propidium iodide emits in the red/yellow range and can be used with a krypton/argon laser. The quality of DNA staining is similar to that achieved with To-Pro-3.

1. Use the same procedure as for To-Pro-3 except put 2.5 µ*M* propidium iodide in TBSB.

3.4. Detection of GFP in Living Worms

GFP is routinely used as a reporter for looking at the localization of gene products in living worms. It can be readily viewed in living or fixed worms using the fluorescein isothiocyanate (FITC) filter sets on the confocal microscope.

1. Using a pick, place worms onto an agarose pad of 4% agarose in dH_2O *(7)*.
2. Add 8 µL of M9 containing 0.25 mM levamisole to immobilize the worms (*see* **Note 21**).
3. Gently put coverslip over the worms.
4. Worms are ready to view.
5. Set up the confocal to view FITC emission. Keep the laser power as low as possible to avoid damage to the worms. While viewing, watch for movement and bleaching (*see* **Notes 22–25**).

4. Notes

1. Worms generally do not stick well to slides, so start with plenty of animals and be gentle when doing washes. Use >10 adults or >40 larvae. Some people find it helpful to monitor the worms under a dissecting microscope during the washes.
2. A humidified chamber can be made from a plastic container with a wet paper towel taped to the lid.
3. 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 the adults and larvae off, then scraping the remaining embryos off with a pasteur pipet into

additional M9. The worms or embryos are then pelleted by centrifuging for 1–2 min at 1000 rpm in a microfuge. To remove *E. coli*, more M9 is added and the worms are pelleted again. Six to eight microliters of concentrated worms can then be dropped onto a slide.

4. 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 many large adults (or larvae), it is difficult to permeabilize L1s without completely squashing the adults. It becomes increasingly more difficult to effectively permeabilize the worms as they get older.
5. 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, **Subheading 3.1.2.**).
6. Another commonly used procedure for whole mounts is the reduction/oxidation method of Finney and Ruvkun ***(8)***. This method is described in detail in Miller and Shakes ***(9)***.
7. Variations in fixation protocols. Paraformaldehyde fixation improves morphology; however; it can interfere with antibody binding and can be omitted. Concentrations of between 1% and 5% paraformaldehyde and incubation times of 30 s to 30 min at room temperature in a humidified chamber are commonly used ***(3,10,11)***; adjust time and concentration for ideal staining. Acetone incubation can also interfere with some antibody reactions and can be omitted or shortened. For some antigens, incubation in cold N, N-dimethyl formamide works better than MeOH or acetone (***12***, SLC and Voula Kodoyianni, *unpublished observations*). Finally, for some antigens, rehydration through a series of increasingly aqueous solutions of MeOH ***(11)***, EtOH ***(9)*** and acetone ***(13)*** have been used.
8. Instead of putting pressure on the coverslip, a Kimwipe can be used to wick excess liquid from under the coverslip until the worms flatten.
9. This fix works well for at least some membrane proteins ***(5,14)***, but not for the cytoskeletal proteins actin and tubulin. For cytoskeleton, try fixing first in 100% MeOH for 5 min at room temperature followed by 1% paraformaldehyde for 25 min at room temperature ***(5)***. In addition, other protocols have been used for fixing extruded tissues ***(4,15)*** and/or cytoskeleton ***(4,15,16)***.
10. Levamisole causes the animals to contract, which results in their germ lines and intestines being extruded efficiently.
11. Common background problems are intestine autofluorescence in the DAPI and fluorescein channels, dim nuclear stain, some cuticle fluorescence. If the 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 primary and/or secondary antibodies with worm or bacterial acetone powder or with fixed worms. Null mutants should be used so that the specific antibody will not be depleted.
12. Do not let the worms dry after they have been fixed and rehydrated; this tends to give a nonspecific haze to nuclei and cytoplasms.

13. If the morphology looks poor, try to fix the worms more quickly. Alternatively, contaminated solutions can distort the morphology. DAPI-stained DNA should look well defined and crisp; if it doesn't, be suspicious. Using different fixes or making small changes in concentration of fixative or time of fixation can make a big difference in the quality of staining.
14. Use an antibody that is known to work to test for good morphology, permeability, and fixation. Some useful control antibodies are anti-DNA monoclonal mAb 030 ***(5,10)***, anti-actin clone C4 ***(4,10)***, anti-β-tubulin ***(5,10,15,16)***; anti-phosphohistone H3.
15. Several approaches can be used to test for specificity of staining:
    a. Stain a null mutant and look for lack of staining. If a null mutant is not available, it is possible to eliminate the antigen (Voula Kodoyianni, *personal communication*) in embryos from animals that have been injected with antisense RNA ***(17,18)***.
    b. Compete with proteins that contain the antigen used to raise the antibodies.
    c. Compare patterns obtained with antibodies to different regions of the protein.
16. Alternatively, the slides can be immersed in a coplin jar if the worms are well attached to the slide.
17. To preabsorb with acetone powder:
    a. Add approximately 1 mg of worm acetone powder/200 µL secondary antibody solution.
    b. Incubate the secondary mix at 4°C for 15 min to 1 h.
    c. Centrifuge the mix at 10,000 rpm for 5 min in a microfuge to pellet the acetone powder.
    d. Use the supernatant.
18. Viewing worms on the confocal.
    a. For most samples we use a 63× (NA 1.4) oil immersion lens (**Fig. 1**). In some cases, e.g. if an image of a whole worm is required, it is useful to use a 25× oil immersion lens (**Fig. 2**).
    b. For single focal plane images, we use the standard triple labeling methods provided with the confocal. Use the lowest laser power that gives a reasonable signal. We usually collect double- and triple-labeled images simultaneously. If there is significant bleedthrough from one channel to another, images can be taken sequentially using a single laser line. In addition, if one fluor is brighter than another images can be taken sequentially using different laser powers.
    c. Z-series are useful for recording data in all focal planes. We usually use 8–15 1-µm sections depending on how flat the sample is. This has been useful for looking at the difference in morphology between the surface and the middle of the germ line ***(5)*** and for looking at staining patterns in different focal planes of embryos and larvae.
    d. Projection of Z-series has been useful to show the complete number of nuclei (e.g., Kodoyianni *et al.*, in preparation), P granules ***(19)***, or distal tip cell processes (e.g., Gao *et al.*, in preparation) in a single image.

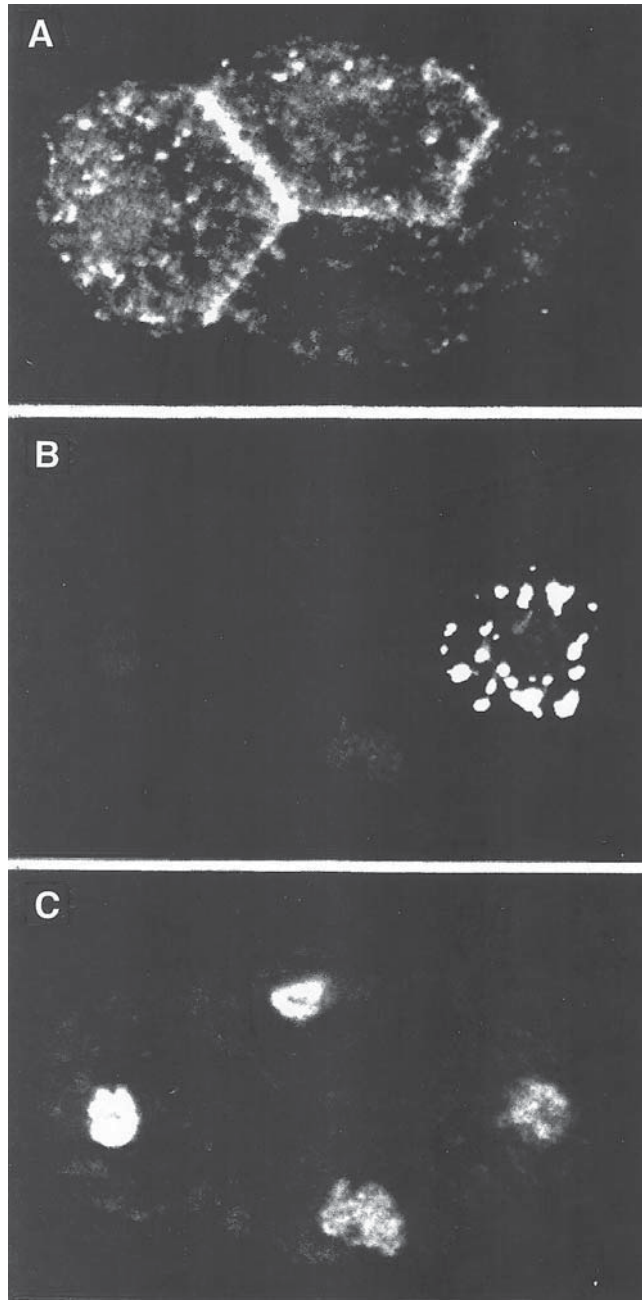

Fig. 1. Four-cell embryo stained with **(A)** anti-GLP-1 antibodies, **(B)** anti-P-granule antibodies, and **(C)** Yo-Pro™-1. Embryos were prepared by freeze-cracking followed by incubation in methanol, then acetone.

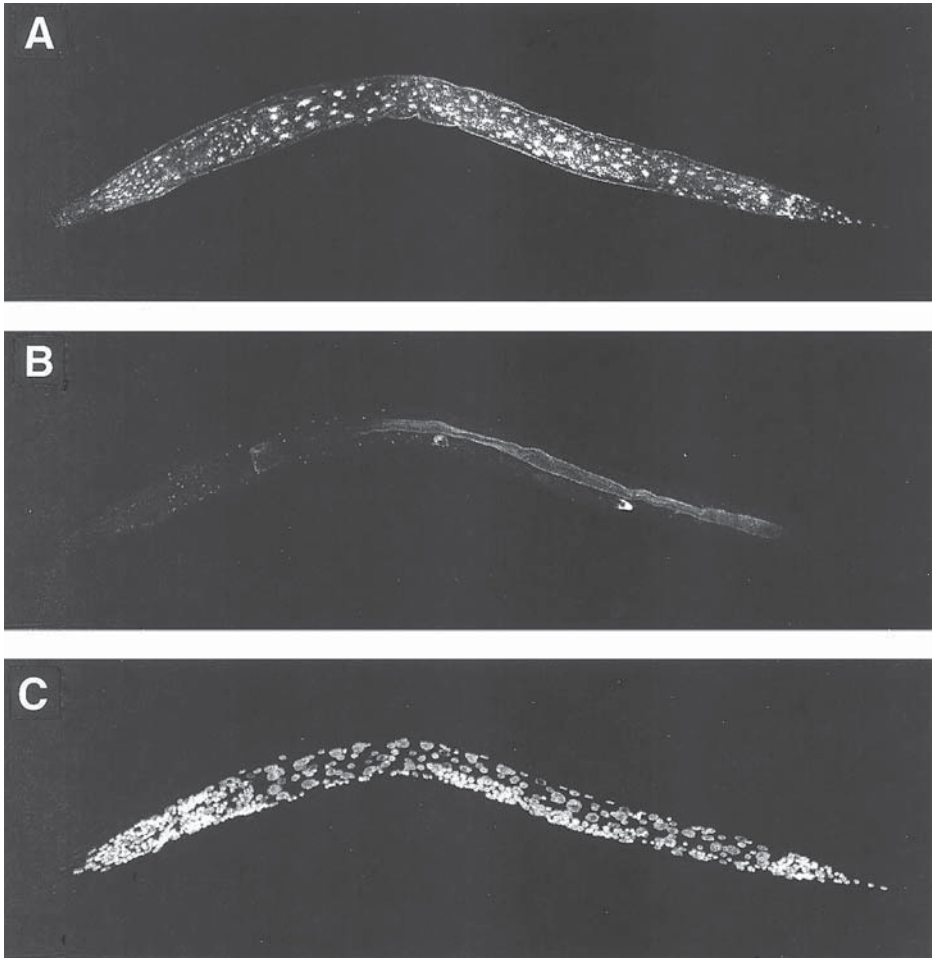

Fig. 2. L3 larva stained with **(A)** anti-LAG-1 antibodies, **(B)** anti-GFP antibodies, and **(C)** To-Pro-3. Larvae were prepared by freeze-cracking followed by incubation in DMF. This animal carries a GFP reporter that is expressed in the two distal tip cells. LAG-1 is a transcription factor expressed in nuclei (Kodoyianni et al., in preparation).

19. Molecular Probes also has other DNA binding dyes. They do not appear to be as clean as To-Pro-3 and propidium iodide; however if To-Pro-3 or propidium iodide do not work well on your sample or if you want a dye that emits in another channel, it may be worth working out conditions for the other dyes.
20. To-Pro-3 is often dimmer than antibody staining. To obtain a good image, try using the far red line of the laser alone and increase the laser power. You can then take sequential images if you have a double- or triple-stained sample. This way the other channels will not be bleached by the high laser power.

21. Levamisole will make the animals contract and look shorter and fatter than usual.
22. It is possible to see very dim (i.e., not visible in the intact animal) GFP fluorescence in tissues extruded from the animal (e.g., gonad, intestine or pharynx; Gao *et al.*, in preparation).
23. GFP fluorescence is detectable in fixed worms; however, it is often dimmer than in live worms. If it is difficult to see, anti-GFP antibody works well. Use a fluorescein-labeled secondary antibody so that both endogenous and antibody-stained GFP are seen in the same channel.
24. Z-series (Gao *et al.*, in preparation) and four-dimensional data sets (Sam Henderson, *personal communication*) of GFP fluorescence can be obtained.
25. With a transmission detector it is possible to take simultaneous Nomarski and fluorescent images. Because Nomarski filters severely cut back the laser power (e.g., we use 100% laser power for Nomarski images) it is often useful to collect an image of GFP first and then to collect the transmitted Nomarski image. The Nomarski image usually is not great.

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