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Fluorescence-Integrated Transmission Electron Microscopy Images

Integrating Fluorescence Microscopy With Transmission Electron Microscopy

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Summary

This chapter describes high-pressure freezing (HPF) techniques for correlative light and electron microscopy on the same sample. Laser scanning confocal microscopy (LSCM) is exploited for its ability to collect fluorescent, as well as transmitted and back scattered light (BSL) images at the same time. Fluorescent information from a whole mount (pre-embedding) or from thin sections (post-embedding) can be displayed as a color overlay on transmission electron microscopy (TEM) images. Fluorescence-integrated TEM (F-TEM) images provide a fluorescent perspective to TEM images. The pre-embedding method uses a thin two-part agarose pad to immobilize live *Caenorhabditis elegans* embryos for LSCM, HPF, and TEM. Pre-embedding F-TEM images display fluorescent information collected from a whole mount of live embryos onto all thin sections collected from that sample. In contrast, the postembedding method uses HPF and freeze substitution with 1% paraformaldehyde in 95% ethanol followed by low-temperature embedding in methacrylate resin. This procedure preserves the structure and function of green fluorescent protein (GFP) as determined by immunogold labeling of GFP, when compared with GFP expression, both demonstrated in the same thin section.

Key Words: *C. elegans*; F-TEM images; high pressure freezing; correlative microscopy; GFP; immunogold labeling; high pressure freezing; AJM-1.

1. Introduction

The pre-embedding method relies on genetic manipulation of *Caenorhabditis elegans* to create a strain with a rescuing array containing green fluorescent protein (GFP). The presence of GFP is used only to identify the genotype of embryos.

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Because *C. elegans* embryos are difficult to fix with conventional chemical fixation, high-pressure freezing (HPF) is the method of choice. The agarose mount we describe for embryos is used for ultrastructural analysis, using freeze substitution with 1% osmium and 0.1% uranyl acetate.

A second postembedding method collects fluorescence images directly from thin sections. We have visualized GFP, red fluorescent protein (RFP), and fluorescent phalloidin staining in 100-nm (thin) sections after low-temperature embedding in methacrylate embedding resin ***(1,2)***. This process can be accomplished using HPF or chemical fixation, but requires a cold dehydration and infiltration protocol that avoids dehydration in absolute solvent and uses a water-tolerant embedding resin. We combine immuno-transmission electron microscopy (TEM) images (anti-GFP immunogold labeling) with native AJM-1::GFP expression from the same thin section. The additional water added to the freeze substitution media to preserve GFP expression does result in some freeze damage.

1.1. Pre-Embedding Fluorescence: Laser Scanning Confocal Microscopy (LSCM), HPF, Epoxy Embedding, and TEM

Embedding embryos within a thin agarose pad allowed us to determine the genotype of embryos by LSCM and examine the same embryos by TEM ***(3,4)***. We used a transgenic strain containing a lethal mutation, *ajm-1(ok160),* rescued to viability with an extrachromosomal array containing wildtype *ajm-1* DNA fused to GFP. Animals with the array express AJM-1:: GFP and are rescued to viability. Embryos without AJM-1::GFP phenocopy *ajm*-1 null mutants develop slowly and arrest as twofold embryos that are approximately twice the length of the egg.

The agarose pad is made of a thin base of high-strength agarose. The thin base layer provides the strength and toughness to keep the mount intact. An agarose pad composed only of low-melting agarose would not hold the pad together well enough to allow transfer to a HPF specimen carrier. Additionally, it is necessary for the top agarose layer to be very thin to allow imaging in a LSCM.

1.2. Postembedding Fluorescence: HPF, Acrylic Embedding, LSCM, and TEM

A complete loss of GFP fluorescence is observed in absolute ethanol ***(5)***, methanol, and acetone. The addition of 1% to 5% water in the freeze substitution mixture has previously been reported to improve visualization of membranes after HPF ***(6)***. Pombo et al. ***(7)*** viewed fluorescently labeled cryosections by LSCM and re-embedded the same sections in Epon for TEM immunogold evaluation of the same section. Luby-Phelps and co-workers ***(8)*** first described the

detection of postembedding GFP fluorescence in one micron plastic (methacrylate) sections. We have found that GFP can be observed directly in plastic thin sections, which is more convenient than cryo-sectioning, and allows examination of the same structures by LSCM and TEM. The acquisition of a backscattered light (BSL) image, which is aligned with, and acquired at the same time as, a fluorescent image, provides surface information to align thin-section fluorescent images with TEM images from the same thin section. TEM images also can be aligned with GFP fluorescent images using patterns of anti-GFP immunogold labeling. This allows "native" GFP fluorescence to be compared with gold labeling observed by TEM. Thin sections can be viewed in a LSCM before TEM to find an area or orientation of interest or to locate a scarce antigen before TEM ***(9)***. The ability to visualize antigen (GFP) in thin or thick sections is useful to confirm the presence of an antigen and to evaluate immunolabeling procedures by light microscopy.

2. Materials

2.1. General Materials Needed for Either Method

1. Glass slides.
2. Glass cover slip, 22-mm square (*see* **Note 1**).
3. High pressure freezing specimen carriers (refer to Chapter 10 in this volume).
4. L-Hexadecene (*see* **Note 2**).
5. High-pressure freezer (we used the BAL-TEC HPM 010).
6. Petri dishes to make a humid chamber (95 and 60 mm diam. dishes).
7. 20-mL scintillation vials for resin infiltration.
8. 3-Aminopropyltriethoxy-silane, 3-APTS (Sigma, A-3648).

2.2. Pre-embedding method

1. Watch glass to cut open hermaphrodites.
2. Dissecting microscope.
3. Mouth pipet.
4. Microcapillary pipets (50 µL) to transfer embryos (*see* **Note 3**).
5. Scalpel to cut open gravid hermaphrodites (*see* **Note 3**).
6. Eyelash glued to a toothpick to move embryos.
7. Agarose (1% gel strength of 1000 g/cm^2 or greater for base; Invitrogen, cal. no. 15510-027; *see* **Note 4**).
8. Low melting temperature agarose (Sigma, A-9539) to immobilize embryos.
9. 2-mL Polypropylene vials with screw cap lids for freeze substitution (refer to Chapter 10 in this volume).
10. Styrofoam box, dry ice, rotary shaker in 4°C cold room (*see* **Note 5**).
11. Disposable polyethylene pipets (Fisher, cat no. 12-711-7; *see* **Note 6**).
12. Microtiter plate shaker and smaller Styrofoam box in –20°C freezer.

13. Single edged razor blade (*see* **Note 7**).
14. Sharpened tooth picks (*see* **Note 7**).
15. Rain-X (Unelko Corp., Scottsdale, AZ) to coat slides for flat embedding (*see* **Note 8**).
16. Velap (equal volumes of petroleum jelly, lanolin and paraffin).
17. Clear acetate tape (Scotch brand or similar) for use as a spacer.
18. Richardson's stain for thick sections (*see* **Note 9**).

2.1.3. Postembedding Method

1. Rotary mixer in −20°C freezer (*see* **Note 10**).
2. Gelatin capsules, size "00" (*see* **Note 10**).
3. Ultraviolet (UV) light (model UVL-56, long wave UV-366 nm, UVP, Inc. San Gabriel, CA) or equivalent.
4. Cardboard box to hold UV light 25 cm above gelatin capsules while in −20°C freezer (*see* **Note 11**).
5. Nickel EM finder grids, 200 mesh honeycomb (Electron Microscopy Sciences [EMS], cat. no. LH200-Ni; coat grids with formvar or other electron stable polymer; *see* **Note 12**).
6. LR-Gold embedding resin with BME (EMS kit including LR Gold and BME cat. no. 14370; *see* **Note 13**).

2.2. Buffers

Use N-Hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES) buffer for agarose (pre-embedding). Make agarose solutions with 0.1 *M* HEPES buffer (*see* **Note 14**).

For Immunolabeling, use the following buffers and reagents (post-embedding):

1. 0.02 *M* Phosphate buffer, pH 7.3 (*see* **Note 15**).
2. Blocking reagent, 0.5% nonfat dry milk (***[10]*** *see* **Note 16**).
3. 20 m*M* Tris buffer (*see* **Note 17**).
4. 225 m*M* NaCl in 20 m*M* Tris buffer (*see* **Note 18**).
5. Primary antibody: Rabbit anti-GFP (Research Diagnostics Inc., cat. #RDI-GRNFP4 abr); anti-GFP antibody comparison is described in Paupard et al. ***(11)***.
6. Secondary antibody: goat anti-Rabbit Lissammine Rhodamine conjugate (Jackson Immuno Research, West Grove, PA, cat. no. 111-085-003. Note: this conjuagate is no longer available).

2.3. Freeze Substitution Medium

1. The freeze substitution medium for pre-embedding method (1% osmium tetroxide with 0.1% uranyl acetate in acetone; *see* **Note 19**).
2. Freeze substitution medium for post-embedding method (1% paraformaldehyde in 95% ethanol) is prepared by adding 0.5 mL of 20% paraformaldehyde (*see* **Note 20**) to 9.5 mL 200 proof ethanol.

2.4. Preparation of 3-APTS-Coated Coverslips to Hold Half Micron Sections for LSCM

Thick sections on 12-mm round coverslips can be immunolabeling with fluorescent secondary antibodies to confirm immunoreactivity and labeling reagents and procedures prior to immunogold labeling. Thick sections are also useful to observe GFP fluorescence prior to examination of thin sections (*see* **Note 21**).

3. Methods

3.1. Pre-Embedding Method for Live Embryos

3.1.1. An Agarose Mount for Live Embryos

1. A thin (high-strength) agarose pad is formed over a standard glass microscope slide. Agarose is dissolved in 0.1 *M* HEPES (neutral pH) buffer to a final concentration of 4 to 5%. The thickness of the pad can be controlled by adding a single layer of cellulose tape over two slides on either side of the slide to be coated. Add 100 µL of melted agarose to the top of the center slide and compress the hot agarose to the thickness of a layer of Scotch tape (approximately 60 µm) with a fourth slide resting on the two adjacent tape-covered slides. Allow the agarose to solidify before sliding the top slide off. Place the slide with the agarose pad in a humid chamber. A humid chamber can be made by placing a 25-mm diameter Petri dish inside a 95-mm Petri dish and adding water to cover the bottom of the large dish.
2. C. elegans embryos are obtained by cutting open gravid hermaphrodites in a watch glass filled with distilled water. The desired age embryos are mouth pipetted to the agar pad and grouped together in the center of the pad using an eyelash glued to the end of a toothpick. Any visible "standing" water on the pad is pulled away from the embryos with the eyelash brush, and allowed to evaporate. Only one group of embryos, no larger than one "confocal field of view," is embedded per slide to minimize exposure to UV light. The use of a 63X objective limits the field of view to approx 10 embryos. Larger groups of embryos are impractical and difficult to navigate in the TEM.
3. Place 70 µL of 5% low-melting temperature agarose dissolved in 0.1 *M* HEPES along one edge of the agar pad. Quickly position a glass cover slip over the low melt agarose to spread it out before it solidifies. Spacers can be used on either side of the slide to obtain the correct thickness. Use the edge of a slide resting on two spacer slides on either side of the slide containing embryos to apply pressure to the cover slip and compress the agarose to a uniform thickness. Ideal mount thickness is 100 µm, which is the thickness of the smallest HPF specimen carrier configuration (*see* **Note 22**).
4. Seal the edges of the slide with hot velap to prevent dehydration; the slides are now ready for confocal microscopy.

3.1.2. Confocal Microscopy of Live Embryos

1. Acquire a focal series through the group of embryos at 1 µm intervals using the appropriate excitation wavelength (488 nm for GFP). A transmitted light image can be acquired simultaneously with each fluorescent image of GFP expression. For a Bio-Rad MRC 1024 we set laser power to 3% and photomultiplier gain to 1500 to maximize the detection of GFP. The developmental stage of the embryos can also be assessed at this time. In our experiments, embryos must attain a minimum age to be able to identify "mutant" from rescued embryos. The transmitted light image can be used to confirm the developmental stage of embryos.
2. We used an image scan size of 512 × 512 pixels. Increasing the image resolution results in a greater light exposure, which is unnecessary for the detection of our specific GFP. Control embryos from the same strain can be embedded in agarose, imaged by confocal microscopy, and filmed by four-dimensional microscopy to verify normal development and viability.

3.1.3. HPF and Freeze Substitution

1. Configure HPF specimen carriers to provide a 100- or 200-µm deep well. To allow access to the agarose-immobilized embryos, scrape the velap off the slide (which has already been imaged in a confocal microscope), and push the coverslip horizontally off the agarose pad using a single-edged razor blade. Cut out a small square of the agarose including the embedded embryos (maximum size is 2 mm diameter) and transfer this small pad to a bottom specimen carrier using the razor blade. The agarose pad can be pushed into a specimen carrier using a sharpened tooth pick that has been coated with 1-hexadecene to keep the tooth pick from adhering to the agarose pad (*see* **Note 23**). The specimen carrier can be filled with bacteria and 1-hexadecene.
2. HPF is preformed in a BAL-TEC HPM 010. As described by McDonald ***(12)***, the filling of specimen carriers is the most important aspect of HPV. Specimen carriers must be full, without air bubbles or voids in order to reach 2100 Bar pressure. The actual freezing is initiated with the press of a button followed by a blast of liquid nitrogen. Within 2 to 3 s, the specimen holder must be removed and rapidly immersed in liquid nitrogen to separate the specimen carriers. The holders are split apart while under the liquid nitrogen, and all tools in contact with the specimen carriers are precooled in liquid nitrogen.
3. The use of a brass bottom and aluminum top (or vise versa) allows for quick identification of the bottom carrier, which is transferred, while under the LN_2, to a polypropylene freeze substitution vial filled with frozen 1% osmium tetroxide and 0.1% uranyl acetate in acetone. The vial is capped and transferred to an aluminum block also cooled in liquid nitrogen.
4. The empty holes in the aluminum block are filled with liquid nitrogen and the block is wrapped in aluminum foil and packed with crushed dry ice in a Styrofoam box taped to a a rotary shaker. The box is shaken at 100 rpm for 3 to 4 d at −80°C (dry ice; *see* **Note 12**).

5. Shake vials on a microtiter plate shaker at 100 rpm for 2 to 3 d at −20°C.
6. Warm to 4°C overnight, transfer to room temperature, and rinse with three to four changes of dry acetone. Agarose blocks can usually be identified by the presence of the embryos.

3.1.4. Epoxy Infiltration and Polymerization for Agarose-Embedded Embryos

1. Transfer freeze substituted specimens into 20-mL scintillation vials (wash and oven dry) containing 30% Epon in acetone (EM grade) rotating on a rotary mixer for 4 hr to overnight at room temperature.
2. 50% and 75% Epon in acetone for 2 h each at room temperature.
3. Three changes of 100% Epon for 1 h each at 50 to 60°C.
4. Transfer the resin-infiltrated agarose pad to a Rain-X or Teflon coated slide with some fresh resin. Place 2 thicknesses of Parafilm on both ends of the slide as a spacer and place a second coated (Rain-X) slide over the Epon-infiltrated agarose.
5. Polymerize on a flat surface in a 60°C oven for 24 to 48 h.
6. Remove the resin from between the slides, rough up one side by scraping a razor blade across the surface, and mount/glue on a blank Epon block for sectioning (*see* **Note 24**).
7. Cut 0.5-µm sections and place on 3-APTS-coated cover slips. Stain thick sections with Richardson's stain. Cut thin and/or semi-thin sections after embryos are detected in thick sections by light microscopy.

3.1.5. TEM

Collect thin sections on formvar-coated slot grids to provide an unobstructed view of an entire section. Stain thin and ultrathin sections for 10 to 20 min in 1% aqueous uranyl acetate, followed by 3 min in Reynolds' lead before viewing in a TEM. Thinner sections may require longer staining times. Large montage images can be collected manually and "stitched together" in Photoshop (Adobe) or collected and montaged automatically with analySIS¨ or similar software. When comparing different embryos within the same mount, it is helpful to make a map using a transmitted light image of the embedded group of embryos. Number the "mutant" and rescued embryos to keep track of higher magnification images. Because a TEM image may be a mirror image of the transmitted light image, flip the image in Photoshop with the same numbering scheme to be prepared for either orientation.

3.1.6. Overlaying Correlative Microscopic Images

LSCM fluorescent and transmitted light images are manipulated with Image J and Adobe Photoshop as described in **Subheading 3.2.6.** A single transmitted light image was chosen from the multiple focal planes acquired based on features of interest that were in focus. The fluorescent image was a brightest point

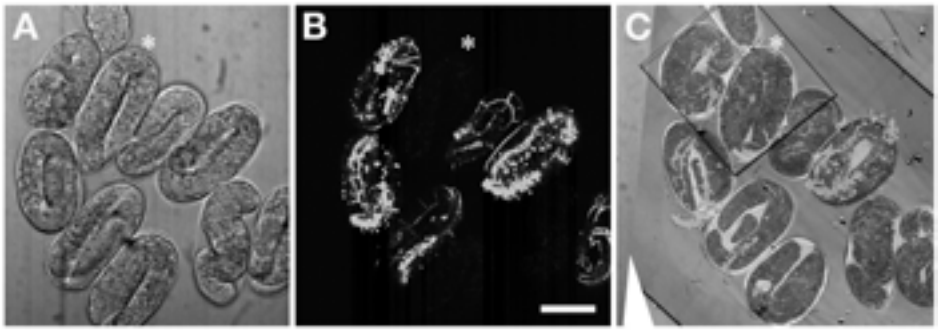


Fig. 1. The same group of *C. elegans* embryos imaged by laser scanning confocal microscopy (LSCM) and transmission electron microscopy (TEM). **(A)** A transmitted light image of live embryos acquired prior to high pressure freezing. **(B)** A brightest point projection of AJM-1::green fluorescent expression used to identify the genotype of individual embryos from a rescued transgenic strain. Scale bar, 20 μm. **(C)** TEM image of the same group of embryos after high-pressure freezing. A white asterisk (for orientation' purposes) marks the same position above a "mutant" embryo in **(A–C)**. The top left highlighted area of **(C)** is shown at higher magnification in **Fig.4A** (in color).

z projection (Image J) of all fluorescent images acquired. Embryos were alive and moving, so fluorescent image(s) may be blurred. The detection of GFP confirms the presence of the rescuing DNA and definitively identifies the genotype of embryos. **Figure 1** demonstrates the pre-embedding correlative method. **Figure 4A** (F-TEM image) integrates pre-embedding fluorescent information with a TEM image.

3.2. Postembedding Methods for Embryos and Adults

3.2.1. HPF and Freeze Substitution

1. C. elegans adults and/or embryos are loaded into specimen holders with bacteria off feeding plates and frozen as described in **Subheading 3.1.3.**
2. After freezing, samples are transferred to vials containing a substitution medium of 1% paraformaldehyde in 95% ethanol on a rotary shaker at 100 rpm in a Styrofoam box for 3-4 d at −80°C (dry ice).
3. Vials are transferred to a smaller Styrofoam box taped to a microtiter plate shaker, (rotating at 100 rpm) for 2 to 3 d in a −20°C freezer.

3.2.2. Infiltration and Polymerization With Acrylic Resin

1. After HPF and freeze substitution samples are infiltrated with 50% and 70% LR-Gold monomer in absolute ethanol each for 8 to 12 h at −20°C.
2. 100% LR-Gold for 12 to 24 h at −20°C.

3. Transfer samples from polypropylene specimen vials to glass scintillation vials in a 4°C cold room (*see* **Note 25**). Three changes in 100% LR-Gold + 0.5% Benzoin Methyl Ether (BME) (two x 8 hr changes and one overnight) at −20°C (*see* **Note 26**).
4. Just before UV polymerization, transfer samples to resin-filled gelatin capsules with precooled tweezers, add tops to gelatin capsules (oxygen will inhibit polymerization), UV polymerize for 15 to 20 h at −20°C.

3.2.3. Sectioning and Anti-GFP Immunolabeling

Thin sections (100 nm) are cut and picked up on formvar coated nickel finder EM grids. Immunogold labeling is conducted on drops on Parafilm in a hydrated chamber with care not to let the grids dry out during any of the immunolabeling steps. We made colloidal gold particles by the citrate reduction method ***(13,14)***. Colloidal particles can be conjugated to a secondary antibody ***(15,16)***. Because the antibody we conjugated is no longer available and commercial probes are readily available, we omit our gold reduction and conjugation methods until they can be confirmed using the currently available antibody. For immunogold labeling:

1. Block, 0.5% non-fat dry milk/ 0.02M PB for 15 min at room temperature.
2. Apply primary (anti-GFP) antibody diluted 1:1000 in 0.02M PB for 1 hr.
3. Rinse in 0.02 *M* PB 3X, then in 20 m*M* TBS 3X.
4. Apply secondary gold conjugate (Goat anti-Rabbit) diluted 1:50 in a mixture of 20 m*M* TBS containing 225 m*M* NaCl for 30 min.
5. Rinse in TBS, rinse in d H_2O, then mount for confocal microscopy.

3.2.4. Confocal Microscopy of Thick and Thin Sections

1. AJM-1::GFP is a brightly expressed GFP that can be observed in fluorescence equipped dissecting microscope. To examine whether a different GFP can be visualized and immunolabeled for TEM, examine the half-micron sections on 3-APTS-coated coverslips by LSCM before the thin sections. Half-micron sections on glass coverslips have five times the sample of a thin section, are flat, and therefore easier for acquiring BSL and fluorescent images. GFP fluorescence will be readily observed in these thicker sections, if they are present in thin sections. Half-micron sections also can be used to work out immunolabeling conditions using fluorescent secondary (detection) antibodies. Red secondary antibodies can be used with anti-GFP primary antibodies, allowing the comparison of immunolabeling to actual GFP fluorescence. GFP signal will be present throughout the entire section, whereas just the surface is exposed for antibody labeling, so secondary antibody fluorescence will be reduced compared with GFP fluorescence.
2. Examples of LSCM images of thick and thin sections of C. elegans worms cut from the same block are shown in **Figs. 2** and **3**. **Figure 2** is single focal plane, BSL image (**Fig. 2A**) and AJM-1::GFP image (**Fig. 2B**) of a thick section on a glass cover slip imaged with a 63X objective. **Figure 3** is a single focal plane, BSL image

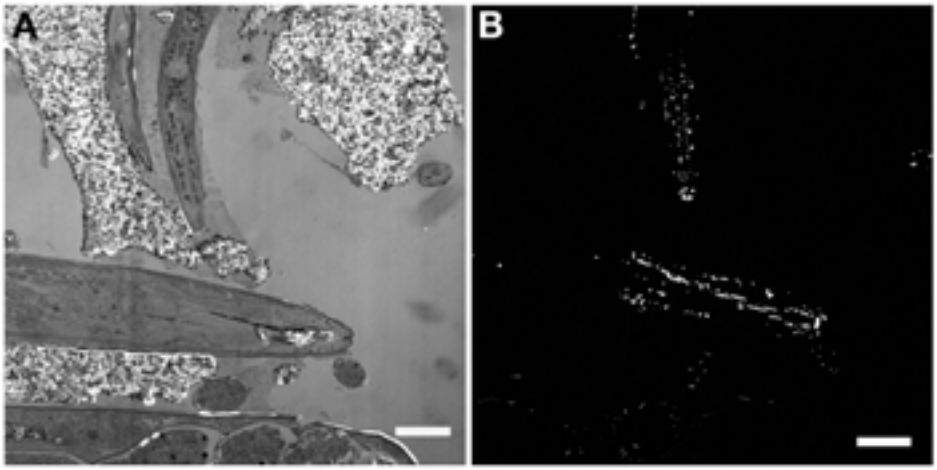


Fig. 2. Postembedding laser scanning confocal microscopy (LSCM) images of a 0.5-μm thick section adhered to a glass coverslip. **(A)** Back-scattered light (BSL) image visualized with a 63x oil objective in a confocal microscope. **(B)** AJM-1::green fluorescent protein (GFP) expression from the same thick section. BSL and GFP images are aligned with each other and acquired simultaneously. Thick sections are useful to confirm the presence of GFP and to optimize anti-GFP immunolabeling conditions by LSCM, before TEM. Bars = 20 μm.

(**Fig. 3A**) and AJM-1::GFP image (**Fig. 3B**) of a thin section on a coated nickel finder grid. These images demonstrate the reduction of GFP signal between thick and thin sections and the difference in BSL image quality when imaging a thick section on a flat surface (glass coverslip) vs a thin section finder grid, which is not flat. Although the z resolution of a LSCM is 500 nm, it may require more than one focal plane to acquire all the fluorescent signals from a thin section on an EM finder grid.

3. Thin sections are imaged by LSCM after immunogold labeling and before fixation and staining for TEM because heavy metal stains will quench fluorescence. For LSCM imaging, an EM finder grid is placed in 20 μL of water in the center of a slide with the section side up. Wet both sides of the finder grid to reduce trapping air bubbles between the grid and coverslip. Seal the edges of the coverslip with hot velap to keep the grid wet. BSL and fluorescent images can be obtained simultaneously with 488-nm excitation. We use a Bio-Rad 1024 LSCM with a 63X oil objective, 3% laser power, and collect 512 × 512 pixel images or 1014 × 768 pixel images. BSL photomultiplier gain is adjusted to obtain the best image of the surface of a thin section. Metal grid bars reflect light as seen in the upper and lower left of **Fig. 3A**. Saturation from metal grid bars is unavoidable when imaging nickel finder grids.
4. Thin sections on EM grids are not flat! Focus must be adjusted often to visualize the surface. The GFP signal is in focus when the BSL image is in focus, so the BSL

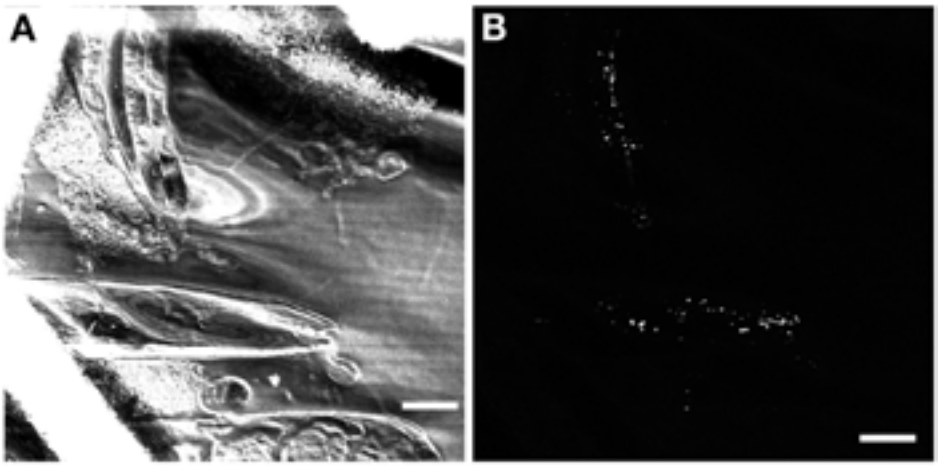


Fig. 3. Postembedding laser scanning confocal microscopy (LSCM) images of a 100-nm thin section. The same worms visualized in the thick section of **Fig. 2** are now visualized in this thin section. **(A)**. A back-scattered light (BSL) image; **(B)** AJM-1::green fluorescence protein expression after high-pressure freezing and low-temperature embedding in methacrylate resin. BSL and fluorescent images are useful to visualize the same structures by LSCM and transmission electron microscopy (TEM) and to align (integrate) fluorescent information from the same thin section with TEM images. Bars = 20 μm.

image provides assurance that focus has been maintained when navigating across areas without fluorescent signal. Because the focal plane changes across a thin section, acquiring a stack of images at different focal planes is useful to acquire all the fluorescent information, especially at lower magnification, which covers a larger area. For the collection of GFP signal, avoid collecting saturated images; low-contrast images are preferable to high-contrast images, Because they can contain more information. Fluorescent image quality becomes increasingly important when images are enlarged 30 times and overlaid onto a TEM image, such as in the **Fig. 4B** inset image (top right).

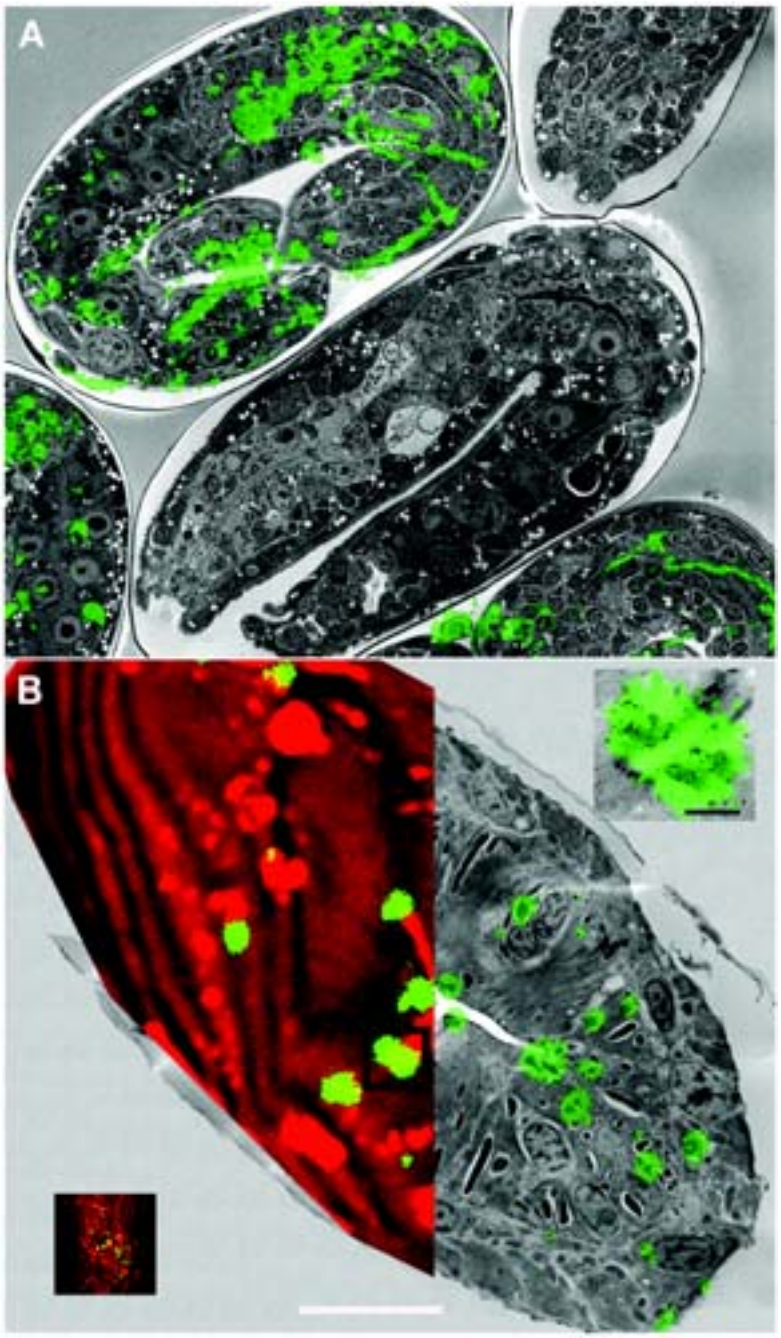
3.2.5. TEM

After LSCM, methacrylate thin sections on Ni finder grids are fixed in 1% to 2% glutaraldehyde in PBS for 5 to 10 min, rinsed in dH_2O, dried, and stained in 0.5% aqueous uranyl acetate for 2 to 4 min and in Reynolds' lead citrate for 1 to 2 min before viewing in a Philips CM120 TEM operating at 60 to 100 kV.

3.2.6. Aligning LSCM and TEM Images From the Same Thin Section

LSCM images were prepared using ImageJ (an open-source image manipulation and analysis program available for free downloading at http://rsb.info.nih.

4-C



4-C

gov/ij/download.html, and Photoshop Elements (Adobe Software). Some of the light (LSCM) and EM images collected may be mirror images of each other; they can be corrected in Photoshop, command sequence Image_Rotate_Flip Horizontal. LSCM and TEM images are aligned starting with the TEM image, which usually contains more pixels; the LSCM BSL and GFP images are merged using ImageJ, Image_Color_RGB Merge command; the BSL image or image stack can be displayed in red and GFP expression in green. The BSL image also can be displayed in gray or blue; however, red provides good contrast to highlight GFP expression. Red and green image stacks are projected using ImageJ, command sequence Image_Stacks_Z project_Max intensity, with the resulting images saved as a TIFF file. This color projection or an image from a single focal plane is opened in Photoshop and pasted over a TEM image, ideally a montage composed of several higher magnification images stitched together to provide an image of the entire worm or cross section. TEM montages provide both low and intermediate magnification perspectives, which are useful for aligning with combined BSL and fluorescent images and for aligning higher magnification fluorescence with TEM images. The center image in **Fig. 4B** is a TEM montage of many images, roughly 4000 by 4000 pixels. A projected BSL (in red) and GFP (green) merged image is shown in the lower left. This image is enlarged and rotated (free transformed proportionally in Photoshop) to match the underlying TEM image. The BSL-GFP image overlies the left half of the TEM montage, whereas just the GFP signal overlies the entire TEM image.

4-C

Fig. 4. *(Opposite page)* **(A)** Shown is a pre-embedding fluorescence-integrated transmission electron microscopy (F-TEM) image, integrating green fluorescent protein fluorescence, as a green overlay on a TEM image. The TEM image is a montage of many TEM images stitched together. The center embryo without a green overlay is the same embryo marked with the white asterisks in **Fig. 1 A–C**. Field of view is 50 μm. **(B)** Shown is an example of a postembedding F-TEM image combined with anti-GFP immunogold labeling. A total of 30 separate TEM images are stitched together to form this TEM cross section of a *C. elegans* worm. This sample was HPF and freeze substituted in 1% paraformaldehyde in 95% ethanol and low temperature embedded in LR Gold. Lower left image combines a BSL image (red) and GFP expression (green) from this same thin section; actual size is 512×512 pixels. This combined BSL and GFP image is free transformed and overlaid on the left half of the TEM image. A combined BSL and fluorescent image is useful to align LSCM and TEM images. Once fluorescent information is aligned with the TEM image, it can be displayed as a green overlay, as seen in the right side of this image. White scale bar equals 5 μm. A F-TEM image of the boxed area near the center of the image is shown at higher magnification in the top right. This image integrates GFP expression from this same thin section and displays it as a green overlay on the TEM image. 25-nm colloidal gold particles label anti-GFP primary antibody. Nominal magnification, ×50,000. Black scale bar = 500 nm.

The BSL image is useful to find the same sample in the TEM and to align fluorescence with TEM images. The top right image in **Fig. 4B** is a F-TEM image acquired at 50,000X nominal magnification with GFP expression shown as a 30% transmittance green overlay. The higher magnification GFP overlay is aligned using the TEM montage for reference. The combined BSL and GFP images are used to accurately align LSCM and TEM images; a separate layer with just the fluorescence signal is usually the only layer displayed over the TEM image. The color overlay is usually at reduced opacity so as not to obscure underlying gold labeling or other TEM details.

4. Notes

1. Although coverslips of other sizes will work, a standard microscope slide is 25-mm wide. A 22-mm wide coverslip provide 1.5 mm of space on each side to seal the coverslip to the underlying slide before velap coats the edges and bottom of the slide. To position embryos in the center of a slide, we draw a small circle in the center on the back side of the slide. Embryos can be positioned in the center of the slide and agarose pad while working with a dissecting microscope. This black circle also is useful when covering the embryos with low melting temperature agarose and pushing down on the coverslip. The circle is wiped off with ethanol prior to confocal microscopy.
2. L-hexadecene is used to coat the top specimen carrier to promote release of carriers after freezing and as filler in the bottom carrier as described by McDonald ***(12)*** and Chapter 8 in this volume.
3. Most *C. elegans* worms are hermaphrodites, with an occasional male. Embryos are routinely collected by cutting open gravid hermaphrodites using a scalpel. Embryos and worms are transparent, so that mature worms containing embryos are readily identified in a dissecting microscope. Once embryos have been liberated from hermaphrodites in a watch glass filled with water, the optimal age or stage of embryo can be isolated with a hair glued to the end of a tooth pick. Embryos are roughly 20 μm by 50 μm in size and can be transferred to the agarose pad using a microcapillary pipet that has been heated and pulled to reduce the orifice size. Embryos are collected using the capillary action of the pipet, with a minimum amount of water and ejected in the center of the agarose pad using a mouth pipet.
4. The strength of agarose can be tested empirically by making a thin pad of agarose over a slide as described in **Subheading 3.1.1.** If the entire pad can be lifted off the slide without tearing, using a razor blade under one corner of the pad, it is strong enough to be used as a base for the correlative pad. We start with a 5% solution of agarose and dilute to between 4 and 5% with additional 0.1 *M* HEPES as needed to form the base pad.
5. Freeze substitution using a Styrofoam box has been described by McDonald ***(12)***. We fit the aluminum block into a tight fitting piece of Styrofoam, which holds the block in place as the dry ice sublimes. We do not monitor the temperature during freeze substitution.

6. Any 3 mL or smaller disposable pipet with its own bulb should suffice. The use of disposable pipets for dispensing fixatives avoids contaminating more expensive pipets.
7. A single-edged razor blade is used to cut out a small piece of agarose containing the embryos to fit within the 2 mm specimen holder. The edge of a razor blade or a fine tipped weighing spatula can also be used to transfer the agarose pad to the specimen carrier. The agarose pad

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8. Two standard 1 × 3-inch microscope slides are coated with Rain-X or Teflon release agent using a cotton tipped applicator or Kimwipe. Coat each slide 3X and buff clear with a Kimwipe. This coating prevents the epoxy resin from gluing the slides together.
9. Richardson's Stain ***(17)***: Make: A. 1.0% methylene blue in 1% borax (w/v in dH_20) and B. 1% Azure II in dH_20. Mix equal volumes of A and B and apply to sections with a syringe equipped with a syringe filter to remove precipitates. Rinse coverslips gently after staining with distilled water.
10. A rotary mixer in a −20°C chest freezer provides good mixing while maintaining a low temperature during resin changes. To hold No. 00 gelatin capsules in a −20°C freezer for UV polymerization: we cut Eppendorf tubes in half and place the gelatin capsules inside the bottom half of the tubes. The sides of the gelatin capsules rise above the cut tubes so they can be capped with the other half of the gelatin capsule after filling with resin and sample. The Eppendorf tubes are held upright in a plastic test tube rack.
11. A cardboard box lined with aluminum foil with a hole cut out for the light source is used to distance the UV light source roughly 25 cm above the gelatin capsules. The technical data sheet provided with the resin also describes polymerization with a Thorn projector lamp (A1/209 FDX, 12V, 100W).
12. Finder grids are marked so that a specific location can be documented with a letter and number. This is helpful, but not necessary, especially if just one section is placed on a grid. In some TEMs, the outer perimeter of a grid may not be accessible, so placing a section in the center of a grid is important to assure access by TEM and will save time in a LSCM looking for the section.
13. LR Gold resin has very low viscosity and is formulated to penetrate biological tissue. Care should be taken to minimize skin contact, especially if there is a history of an allergy to methacrylates.
14. A stock solution of 1.0 *M* HEPES buffer is prepared by the addition of a solution of HEPES acid to a solution of HEPES base (sodium salt).
    13.01 g of HEPES sodium salt is added to 50 mL of dH_2O.
    23.8 g of HEPES acid is added to 100 mL of dH_2O.
    Add the acid solution to the base while stirring until pH = 7.3. Dilute to 0.1 *M*.
15. To prepare 100 mL of 20 m*M* Pb, add 0.045 g of sodium phosphate monobasic (anhydrous) and 0.435 g of sodium phosphate dibasic heptahydrate to 100 mL of dH_2O pH to 7.4. (Use for block and for primary antibody dilutions).

16. For 1 mL of 0.5% blocking solution: add 0.005 g of nonfat dry milk to 1 mL of 20 m*M* phosphate buffer, warm to dissolve; spin for 3 min at 1500 rpm in an Eppendorf microfuge.
17. To prepare 100 mL of 20 m*M* Tris buffer, pH 8.2 at 25°C: add 0.142 g of Tris HCl and 0.134 g of Tris base to 100 mL of dH_2O, pH to 8.2.
18. To obtain 225 m*M* NaCl in 20 m*M* Tris; add 0.131 g of NaCl to 10 mL of 20 m*M* Tris buffer.
19. McDonald ***(12)*** has previously described the procedures to make various freeze substitution mixtures. Briefly, to prepare 25 mL of 1% osmium with 0.1% uranyl acetate, cool 24 mL of EM-grade acetone in a disposable 50 mL of polypropylene tube on crushed dry ice. If 0.5 grams of pure OsO_4 crystals are not consolidated in the bottom of the vial, freeze the unopened vial of solid osmium in liquid nitrogen. Osmium crystals will fall to the bottom of the ampule. Add 1 to 2 mL of the cold acetone to the ampule, mix, and add back to the 50-mL tube of acetone on dry ice. Repeat until all of the osmium is dissolved in the 25 mL of acetone. Add the UA in methanol (0.025g UA in 1 mL of methanol) to the acetone, keep cold on dry ice. Add 1 mL of freeze substitution mix (1% osmium tetroxide with 0.1% uranyl acetate in acetone) to each 2 mL substitution vial and freeze in liquid nitrogen.
20. For 10 mL of 20% paraformaldehyde: 2.0 g of paraformaldehyde is added to 8.5 mL of dH_2O; vortex, heat in a 60°C water bath for 5 min, add 0.2 mL of 0.1 *M* NaOH, and then add water to a total volume of 10 mL.
21. To coat 12-mm round coverslip with 4-APTS:
    Clean round 12 mm round coverslips in detergent solution. Rinse 10x in dH_2O.
    Dehydrate using three changes in absolute ethanol.
    Coat slides in 2% solution of 3-APTS in dry acetone for several minutes.
    Rinse twice in dH_2O.
    Spread out coverslips on clean filter paper and dry at room temp.
22. The most difficult step is covering the embryos with the hot low-melting temperature agarose. This takes practice! This does not work with embryos that have been bleached, because they are too fragile. If visible water is removed the embryos usually do not move as the agarose spreads out over the pad. To obtain agarose of the right consistency, heat the low-melting temperature agarose to boiling in a glass test tube. The agarose is most easily dispensed by cutting 5 to 10 cm off the end of a yellow pipet tip and pre-heating the tip by rotating in the hot agarose. Fill the tip with 70 µL of hot agarose and then dispense the agarose in a line along the edge side of the slide, over the high strength agarose. The actual volume of agarose dispensed on the pad is less than 70 µL, as about half (or more) of the agarose stays in the tip. Use the 70 µL as a starting point and adjust as needed. This small volume cools rapidly, so the coverslip must be quickly placed over the low-melting temperature agarose and gently pressed down to spread a thin layer of agarose around the embryos. A microscope slide turned on edge can be used to apply pressure to the coverslip on either side of the embryos but not directly over the embryos. The top layer of agarose is thinner than the high melting temperature agarose used to make the base. If the mount is too thick, embryos will be beyond the focal depth of the confocal microscope, making it impossible to acquire a fluorescent image.

23. Loading tiny samples into specimen carriers becomes a battle with surface tension. Adhering carriers to the tops of Petri dishes with double stick tape holds carriers in place during the loading process. Double stick tape is available in two flavors, "permanent" and a less aggressive "removable" (Scotch 667 from 3M). The less aggressive tape holds carriers without having to fight to remove them for loading in the freezing holder, just prior to freezing.
24. One side of the now polymerized agarose pad containing embryos is roughed up to remove residual Rain-X or Teflon release agent and to increase the surface area for adhesion to an Epoxy blank. The small sample piece can be attached to the blank using glue or additional Epoxy resin followed by polymerization in a 60°C oven. This orients the embryos parallel with the cutting plane to obtain a similar orientation in the TEM as the view already obtained by LSCM.
25. After freeze substitution in ethanol and paraformaldehyde worms, are usually still inside the bottom specimen carrier. Because the worms have not been exposed to osmium or tannic acid, they are translucent and can be difficult to see. To remove worms from the carriers, we use a dissecting microscope equipped with a light source to illuminate the inside of specimen holders. Ideally, we would remove worms from the holders while at –20°C. Because a walk-in freezer is not available, we use a 4°C cold room and dissecting microscope to remove samples from inside holders and transfer them to scintillation vials. With two pairs of cooled forceps, one holds the specimen cup and a second EM-grade (fine point) forceps scoops out the worms. Bacteria were used as a filler to help hold worms together; because of the light fixation, samples are only loosely held together and require gentle handling.
26. To remove resin from scintillation vials during resin changes without discarding the worms; we use a yellow pipet tip wedged on the tip of a disposable plastic pipet. The small orifice becomes plugged with larger pieces of sample and reduces the chance of discarding sample during resin changes.

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References

1. Sims, P. A. and Hardin, J. D. (2005) Visualizing green (GFP) and red (DsRed) fluorescent proteins in thin sections with laser scanning confocal and transmission electron microscopy, in *Microscopy and Microanalysis 2005*, vol. 11. Cambridge University Press, Honolulu, pp. 6–7.

2. Sims, P., Albrecht, R., Pawley, J., Centonze, V., Deerinck, T., and Hardin, J. (2006) When light microscopy is not enough: Correlative light microscopy and electron microscopy, in *Handbook of Biological Confocal Microscopy*, 3rd ed. (Pawley, J., ed.), Springer, New York, pp. 846–860.
3. Koppen, M., Simske, J. S., Sims, P. A., et al. (2001) Cooperative regulation of AJM-1 controls junctional integrity in *Caenorhabditis elegans* epithelia. *Nat. Cell Biol*. **3,** 983–991.
4. Simske, J. S., Koppen, M., Sims, P., Hodgkin, J., Yonkof, A., and Hardin, J. (2003) The cell junction protein VAB-9 regulates adhesion and epidermal morphology in *C. elegans*. *Nat. Cell Biol.* **5,** 619–625.
5. Ward, W. W. (1998) *Green Fluorescent Proteins: Proteins, Applications and Protocols*, 1st ed., Wiley, New York.
6. Walther, P. and Ziegler, A. (2002) Freeze substitution of high-pressure frozen samples: the visibility of biological membranes is improved when the substitution medium contains water. *J. Microsc*. **208,** 3–10.
7. Pombo, A., Hollinshead, M., and Cook, P. R. (1999) Bridging the resolution gap: Imaging the same transcription factories in cryosections by light and electron microscopy. *J. Histochem. Cytochem*. **47,** 471–480.
8. Luby-Phelps, K., Ning, G., Fogerty, J., and Besharse, J. C. (2003) Visualization of identified GFP-expressing cells by light and electron microscopy. *J. Histochem. Cytochem*. **51,** 271–274.
9. Robinson, J. M., Takizawa, T., Pombo, A., and Cook, P. R. (2001) Correlative fluorescence and electron microscopy on ultrathin cryosections: bridging the resolution gap. *J. Histochem. Cytochem*. **49,** 803–808.
10. Kaur, R., Dikshit, K. L., and Raje, M. (2002) Optimization of immunogold labeling TEM: an ELISA-based method for evaluation of blocking agents for quantitative detection of antigen. *J. Histochem. Cytochem*. **50,** 863–873.
11. Paupard, M. C., Miller, A., Grant, B., Hirsh, D., and Hall, D. H. (2001) Immuno-EM localization of GFP-tagged yolk proteins in *C. elegans* using microwave fixation. *J. Histochem. Cytochem*. **49,** 949–956.
12. McDonald, K. (1999) High-pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling, in *Electron Microscopy: Methods and Protocols*, 1st ed (Hajikbagheri, M. A. N., ed.), *Methods in Molecular Biology*, vol.117 (Walker, J., ed.), Humana Press, Totowa, NJ, pp. 77–97.
13. Slot, J. W. and Geuze, H. J. (1985) A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol*. **38,** 87–93.
14. Slot, J. W. and Geuze, H. J. (1981) Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol*. **90,** 533–536.
15. Simmons, S. R., Sims, P. A., and Albrecht, R. M. (1997) Alpha IIb beta 3 redistribution triggered by receptor cross-linking. *Arteriosclerosis, Thrombosis & Vascular Biol*. **17,** 3311–3320.
16. De Roe, C., Courtoy, P. J., and Baudhuin, P. (1987) A model of protein-colloidal gold interactions. *J. Histochem. Cytochem.* **35,** 1191–1198.
17. Richardson, K. C., Jarrett, L., and Finke, E. H., (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Tech.* **35,** 313.