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Integrating Light and TEM Information with F-TEM images

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Fluorescent fusion proteins are widely used to visualize the localization of proteins in worms, fish, flies and tissue culture cells. We have used two different methods that use high pressure freezing (HPF) combined with correlative light microscopy (LM) and TEM. The first method uses fluorescence from live organisms immobilized in agarose followed by HPF and standard freeze substitution in dry solvent with osmium. This pre-embedding method optimizes ultrastructural preservation. A second, post-embedding method preserves fluorescence and immunoreactivity from embedded and polymerized thin sections. Here we describe post-embedding fluorescent integrated TEM images (F-TEM). Post-embedding F-TEM images combine fluorescence information from a thin section, as a color overlay on a TEM image of the same (100 nm) section. Although correlative immunofluorescence has been demonstrated using thin cryo-sections [1,2], and green fluorescent protein (GFP) has been visualized in 1 μm sections [3], the detection of bioluminescent markers such as GFP or mRFP in methacrylate thin sections has not been previously reported. We demonstrate post-embedding fluorescence of GFP and mRFP in thin sections for correlative light and electron microscopy. The use of plastic embedded material has advantages over cryo-sections, including ease of sectioning without specialized equipment and the ability to generate higher contrast images [3]. We have optimized fixations to preserve fluorophore expression of several *C. elegans* junctional proteins fused to GFP and more recently, to mRFP.

Why obtain fluorescent information from thin sections?

Fluorescent signal from light microscopy provides additional information not obtained by TEM imaging alone. Visualizing fluorophores in plastic embedded thin sections is useful for low and high resolution localization, to find an area or orientation of interest by light microscopy prior to electron microscopy, and for correlative visualization of the same structures by both laser scanning confocal microscopy (LSCM) and TEM. Viewing fluorescent expression in thin sections by LSCM permits higher z resolution imaging than would otherwise be possible, because thin sections are 100nm thick, while LSCM z resolution is 500 nm [4]. Additional benefits of thin sections include sharper images and decreased background fluorescence when imaging with LSCM [1]. Moreover, combining fluorescence microscopy with TEM (F-TEM) can be used to identify genotypes, confirm patterns of immunogold labeling in the TEM, and serve as a reference for framing TEM images in the familiar context of a fluorescent image. Although the GFP present in thin sections is typically assessed by its direct fluorescence, polyclonal anti-GFP antibodies can detect GFP even if it fails to become fluorescent or has denatured, which enhances their utility for labeling weakly fluorescent GFPs [5].

Visualizing GFP facilitates interpretation of antibody labeling by LM

TEM immunolabeling can be frustrating. The use of thick sections and light microscopy labeling with fluorescent secondary antibodies is a rapid way to troubleshoot immunolabeling conditions prior to TEM [6]. The presence of GFP confirms the presence of the target antigen. Optimal labeling will be viewed as the overlap of the two different fluorophores. Because only the surface of the section is accessible to antibodies, there will typically be more GFP signal (from the entire depth of the section) than that provided by secondary antibody fluorescence. Indeed, metal shadowing of Lowicryl thin sections indicates a surface penetration in the 2-6 nm range [7]; although we use LR Gold resin, penetration depth is likely similar.

Viewing sections by LSCM

We evaluate blocks for GFP fluorescence using 0.5 μm sections on 3-APTES coated coverslips. Both thin and thick sections are viewed hydrated in dH_20 and coverslipped. A LSCM is used to visualize both GFP expression and surface information using backscattered light (BSL). 3% laser power has been sufficient to detect several junctional GFP fusion proteins. We routinely scan sections using a 63X oil objective at a resolution of 512 x 512 pixels, using a BSL image to locate samples within sections and to remain focused on the surface of the grid while checking for fluorescence. Photomultiplier gain is initially set at maximum and reduced to avoid collecting images with too much contrast. Weak signal can be enhanced by accumulating photons from multiple scans. The BSL image is simultaneously obtained and aligned with the fluorescent

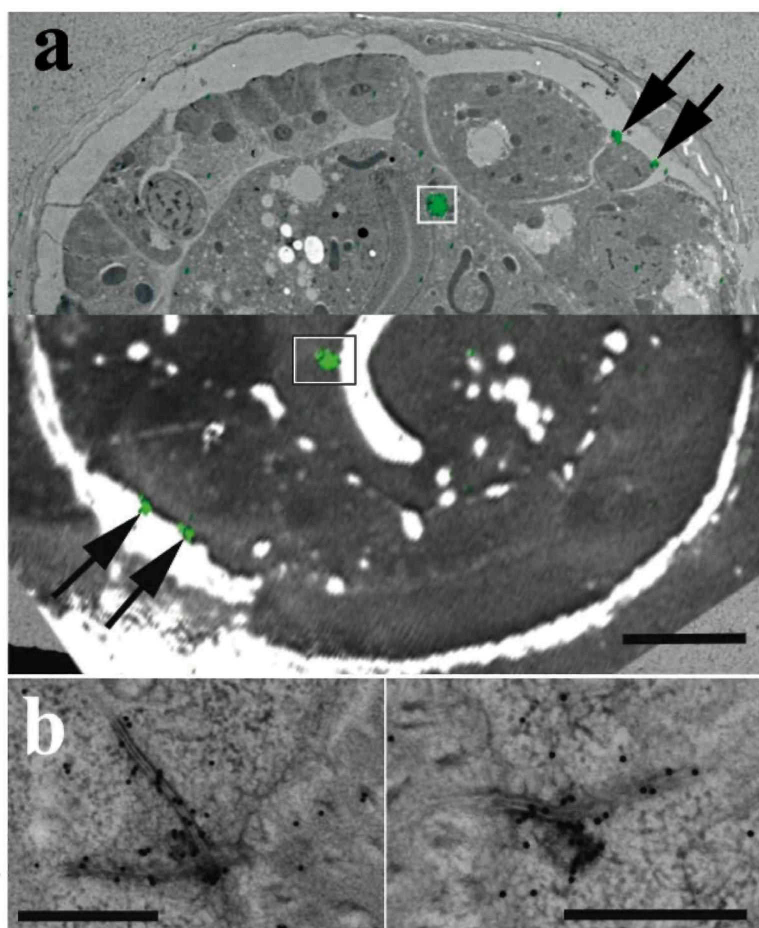

FIG. 1 (a) A thin section, top half imaged by TEM, bottom half imaged by LSCM BSL. AJM-1::GFP(LSCM) shown in green on both images. Black arrows point to seam cell AJM-1::GFP. White boxes are two intestinal apical junctions shown in fig. (b). Scale bar = 5 μm.

(b) Anti-GFP immunogold (20 nm) labeling of intestinal junctions. Scale bars = 500 nm for each image.

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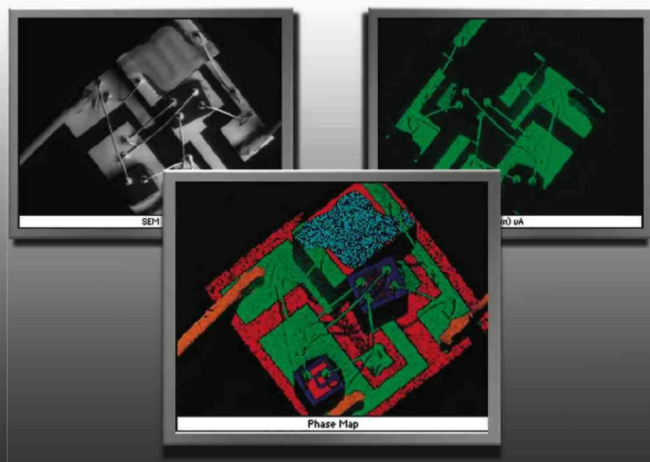

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after light microscopy and stained for TEM in 0.5% aqueous uranyl acetate and lead citrate for 3 minutes and 1 minute, respectively. The use of tannic acid and heavy metal stains quench GFP fluorescence and should be limited or avoided prior to light microscopy.

BSL and fluorescent images can be merged together and /or colored using image processing software, such as Image J or Adobe Photoshop. LM images are free transformed using Photoshop and aligned with TEM images. While free transforming, the x and y axes are locked so that the dimensions of images are varied proportionally. We find it easier to align images if the opacity of an overlaying image is reduced, which allows visualization of parts of two images simultaneously.

Recently, other genetically encoded fluorophores have been described, including a monomeric variant of the red fluorescent protein from coral (mRFP), which shows reduced tetramerization and faster maturation than standard DsRed [11]. We constructed a strain expressing DLG-1::mRFP, which co-localizes with AJM-1 to apical junctions in *C. elegans* epithelia [12]. Using chemical fixation and low temperature embedding in methacrylate resin (LR Gold), we were able to image DLG-1:: mRFP in post embedded thin sections by LSCM (Figure 2). We are currently working to immunogold label mRFP in thin sections. We have also used chemical fixation followed by low temperature dehydration and embedding to visualize fluorescent phalloidin in thin sections.

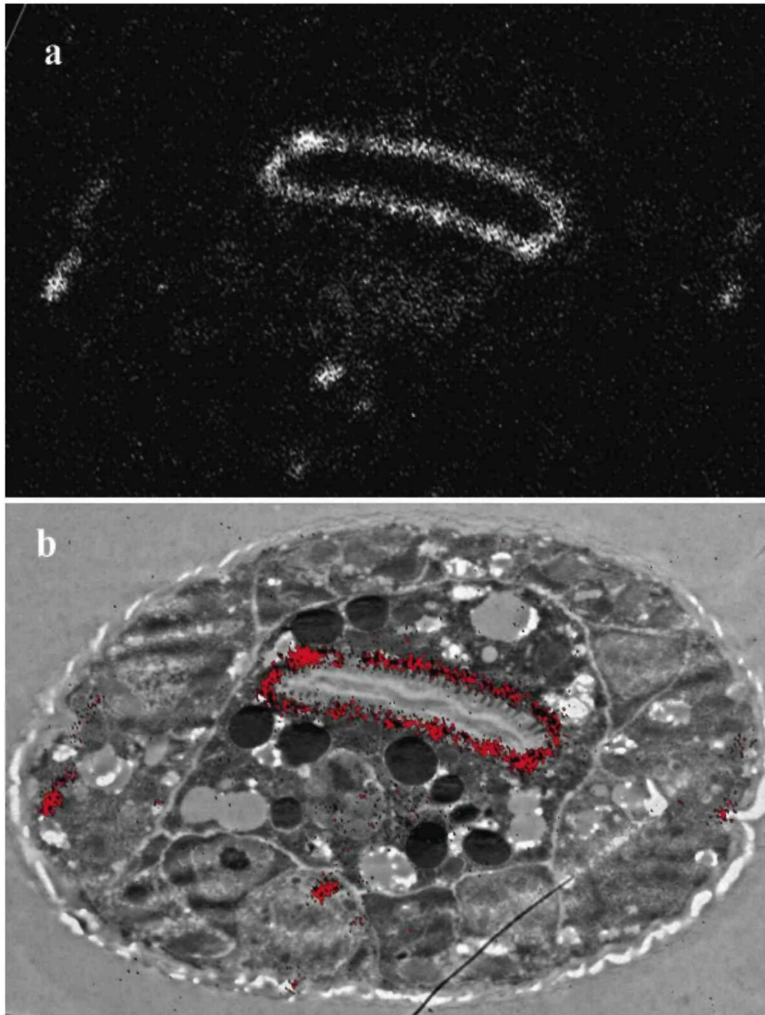

FIG. 2 (a) A contrast-enhanced image of DLG-1:: mRFP acquired using a 63x objective with a BioRad 1024 LSCM (5.9x zoom; field of view is 20 μm). (b) F-TEM image with overlay of red DLG-1:: mRFP outlining the intestine. Red areas in the right and left center are likely marking seam cell junctions. Field of view is 20 μm.

image. The merged fluorescent and BSL image is useful for visualizing areas of fluorescence within the sample in addition to aligning LSCM images with TEM images. TEM of these samples allowed us to correlate immunogold labeling with GFP expression from the same thin section (Figure 1).

High pressure freezing (HPF) *C. elegans* adults followed by embedding preserves fine structure and immunoreactivity and freeze substitution in solvent with 1-5% water has been reported to improve visibility of membranes [8,9]. We combined HPF, freeze substitution, and low temperature embedding to observe AJM-1:: GFP in thin sections and then immunogold labeled the same sections for TEM. AJM-1 localizes to the apical junction in epithelia and can be visualized in the epidermis and intestine. GFP does not fluoresce in absolute solvents [10]. In order to preserve fluorescence, water tolerant embedding resins must be used that do not require dehydration in 100% solvent. We followed the recommended low-temperature dehydration and infiltration schedule for LR Gold using a rotating mixer in a chest freezer.

Figure 1 integrates light and TEM information from the same thin section. The bottom half of Fig. 1a is a BSL image of the surface of a thin section; the GFP signal (green) shows AJM-1::GFP after immunogold labeling. Sections were fixed in 1% glutaraldehyde

Conclusions: the advantages of F-TEM

F-TEM has several advantages. Preparative techniques that optimize preservation of fluorescent information while maintaining the necessary biological structure for TEM or SEM will provide additional information for correlative studies. Various fixation and staining conditions can be rapidly evaluated using fluorescence microscopy, while ultrastructural preservation can be evaluated using EM. In addition, multiple GFP-containing strains can be labeled at the same time in the same section with anti-GFP antibody directly conjugated to colloidal gold. This is possible using strains with unique GFP expression patterns or combinations of mRFP and GFP to differentiate strains with similar GFP localization. Such a one-step technique should be convenient because of its speed and simplicity, and avoids issues of steric hindrance that may arise when using multiple antibodies. Ultimately, F-TEM should become a routine and useful technique in the arsenal of the electron microscopist. ■

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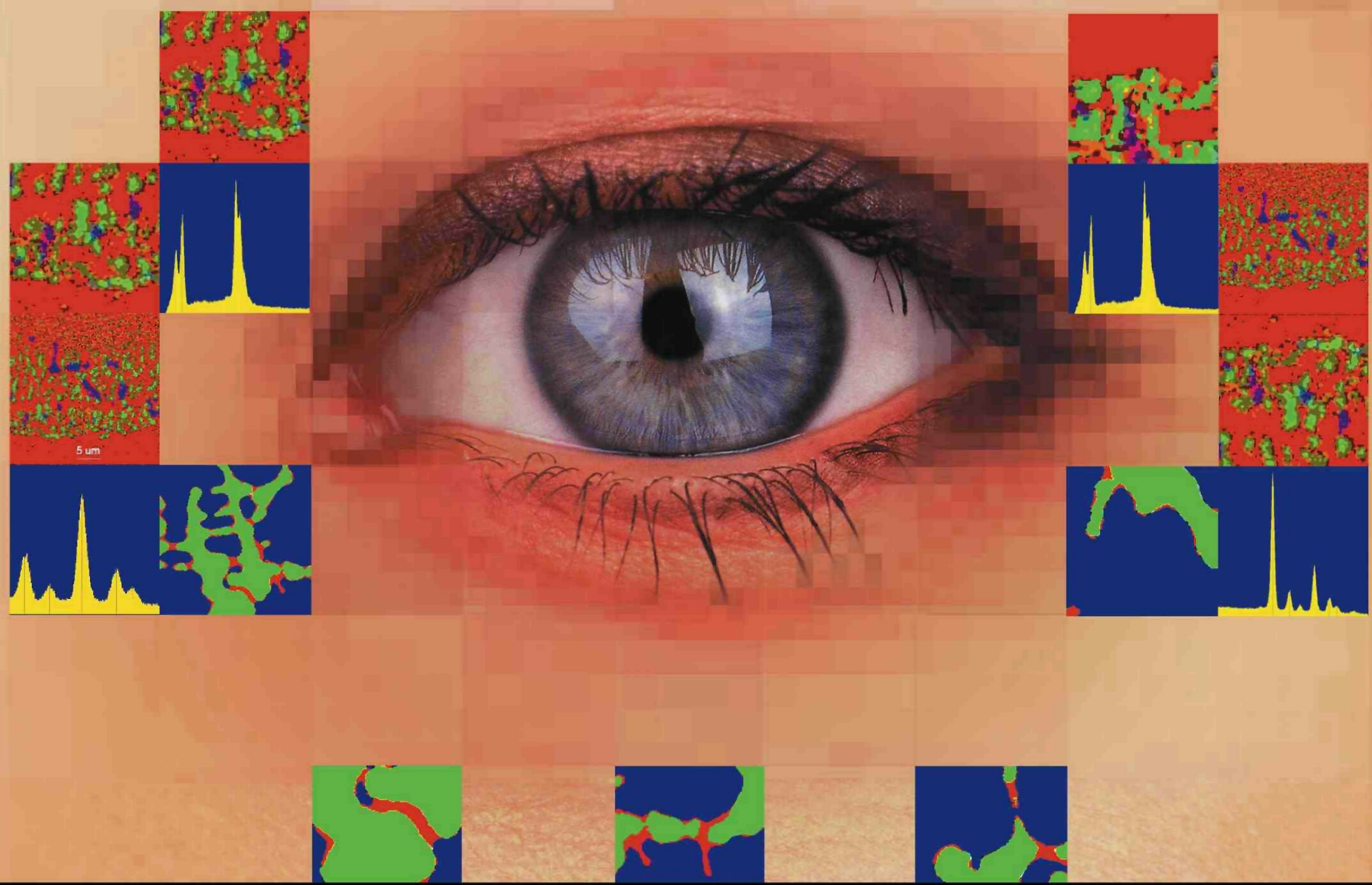

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