

Essentials of Specimen Preparation for High Performance Volume Imaging by SEM

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The unlikely application of scanning electron microscopy (SEM) to 3-D imaging of epoxy embedded specimens has burgeoned over the last few years and will likely eventually supplant conventional TEM as the mainstay of wide-field nanometer-scale cytology of biological specimens. Three different approaches have come to the forefront: Serial block-face SEM (SBEM), whereby a specialized miniature ultramicrotome is fitted into a SEM and the mirror-smooth block-face imaged using backscatter electrons (BSEs) in between automated removal of the surface using a diamond knife; focused ion beam SEM (FIB-SEM), which achieves much the same thing but instead removes the surface material using a focused ion beam; and array tomography SEM (AT-SEM), employing ribbons of serial sections prepared using an ultramicrotome that are mounted on special tape or silicon wafers prior to BSE-SEM imaging. Each of these methods has their own strengths and weaknesses (for review, see [1]), but common to each are the approaches used to prepare biological specimens prior to imaging. Since BSE yield is meager at the low accelerating voltages typically employed (1-4 keV), combinatorial intense heavy metal staining of cells and tissues is needed to maximize BSE yield and improve specimen conductivity in order to minimize specimen charging; important since the samples are embedded in non-conductive epoxy resins. One of the first staining protocols developed specifically to maximize BSE yield from epoxy embedded specimens by SEM employed a variety of previously introduced heavy-metal stains, including potassium ferrocyanide-reduced osmium tetroxide, liganding with thiocarbohydrazide followed by a second osmium tetroxide treatment, and *en bloc* uranyl acetate and lead aspartate staining prior to epoxy embedding [2]. This approach made possible block-face imaging of tissues at high vacuum instead of variable-pressure SEM, vastly improving image acquisition speed and achievable resolution. Recently, a protocol (termed BROPA, for *brain-wide reduced-osmium staining with pyrogallol-mediated amplification*) was developed to achieve homogeneous staining of the entire mouse brain [3], and subsequent modifications have drastically shortened the time required to prepare samples using this method [4]. The ultimate goal of these and future approaches is to try and obtain the same type of image information using these SEM techniques as is currently obtained by ultrathin-section transmission EM (figure 1).

In addition to these protocols, new molecular-genetic imaging probes such as miniSOG [5] and the ascorbate peroxidase derivative APEX [6], as well as new chemical labels [7, 8] have been developed to selectively contrast macromolecules in 3-D with excellent cellular preservation, since they do not require compromises to primary chemical fixation or the use of permeablizing detergents, and are all easily adapted to SBEM, FIB-SEM and AT-SEM (figure 2). These probes and others under development promise to greatly improve the ability for 3-D localization of cellular constituents at high resolution. Furthermore, a method to combine hybrid high-pressure freezing methods with EM-level genetic labeling and heavy metal staining has been introduced, thereby enhancing subcellular morphological preservation of difficult to preserve samples [9]. Finally, the introduction of gas-injection based charge compensation has essentially eliminated specimen charging of samples using SBEM and allows for high vacuum imaging for vastly improved resolution from any sample [10].

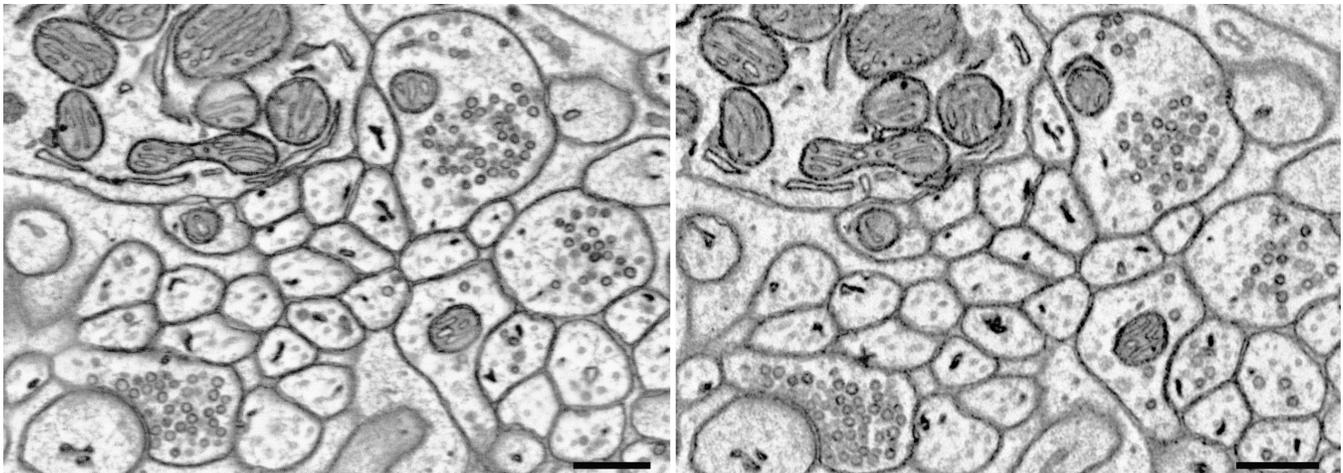


Figure 1. Block-face BSE-SEM image taken at 1.9 keV (left panel) and the corresponding serial ultrathin-section TEM image of the same region taken at 80 keV (right panel) using heavy metal stained brain tissue prepared as described [2]. The images are nearly equivalent. Bars = 0.25 microns.

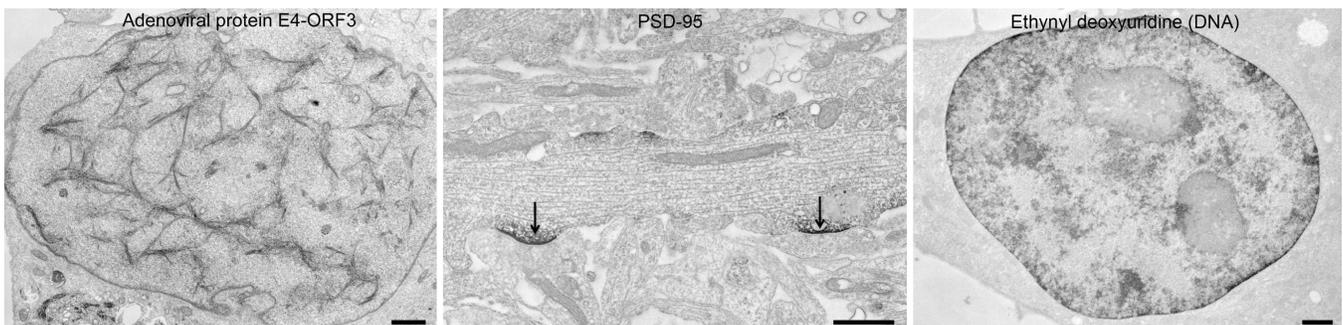


Figure 2. The genetic labeling probe miniSOG (left panel) used to localize the viral protein E4-ORF3 in a cultured cell nucleus, APEX2 used to label the protein PSD-95 (arrows) in cultured hippocampal neuron synapses (middle panel), and click-chemistry labeled DNA incorporated with 5-ethynyl-2'-deoxyuridine (right panel). Bars = 0.5 microns.

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