Three-Dimensional Analysis of Optic Nerve Axons Using a Focused Ion Beam-Based Approach

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Introduction

Neurons in the central nervous system (CNS) are extensively interconnected, and consequently almost half of the human brain is occupied by wiring in the form of the myelinated axons. Dysfunction and degeneration of these axons can result in profound and permanent disability. Axons are much more complex than simple wires, however. Signal propagation relies on elaborate ultrastructural specializations at the nodes of Ranvier, which are demarcated by gaps between myelin sheaths. As living extensions of neurons, axons also require constant replenishment of metabolites and organelles such as energy-generating mitochondria. Studies of pathogenesis of myelinated axons are often constrained by microscopic complexity that requires nanometer-scale resolution over substantial three-dimensional distances. A case in point is the optic nerve. Optic nerve axons are an attractive system for studying CNS axonal dysfunction in animal models of human disease because they are anatomically distinct, they are accessible to experimentation, and the axons are relatively homogeneous in size and function. Optic nerve axons are typically 0.2-3 µm in diameter, with nodes 1-2 µm long that are spaced 100 to 500 µm apart. The axons are densely packed in the optic nerve, with little or no extracellular space between them, and often follow sinuous courses along the nerve. Thus tracing individual axons by confocal microscopy is difficult. Axonal mitochondria are typically 0.1-0.2 µm in diameter and several micrometers long and often exhibit a branched morphology. Light microscopy resolution is insufficient to distinguish between one large mitochondrion and multiple small ones, and in a single TEM section, the same mitochondrion may appear to be one or multiple profiles. Serial sectioning TEM provides the resolution and 3D perspective needed but requires a dedicated investigator with rare patience and cannot be considered a routine technique.

An approach for providing serial TEM-like images of synapses using a focused ion beam (FIB) system was recently described by Knott et al. [1] and suggested a new approach for studying optic nerve axons. An FIB is used to ablate (ion mill) a thin layer from the surface of a plastic-embedded biological specimen, and TEM-like images of the newly milled tissue block face are generated by an SEM operated in backscatter mode. Automated cycles of milling and imaging produce a series of images similar in appearance to TEM serial sections. This method offers several advantages for imaging optic nerves, including automated data collection, imaging axons

in three-dimensional data sets, and the ability to pre-scan the tissue to locate areas of interest. FIB systems are available at many academic institutions as they are widely used for surface analysis, nanotechnology, and microfabrication applications. We set out to investigate whether this approach could be easily implemented for the study of normal and pathological optic nerves. Knott et al. [1] have provided a detailed and informative discussion of the methods for preparing samples and instrument settings for imaging. Here we focus on the factors that are important in implementing a new application and the issues relevant to optic nerve studies.

Preparing Mouse Optic Nerves for Dual Beam FIB Imaging

Tissue preparation for dual beam imaging is very similar to that for conventional TEM of biological samples and
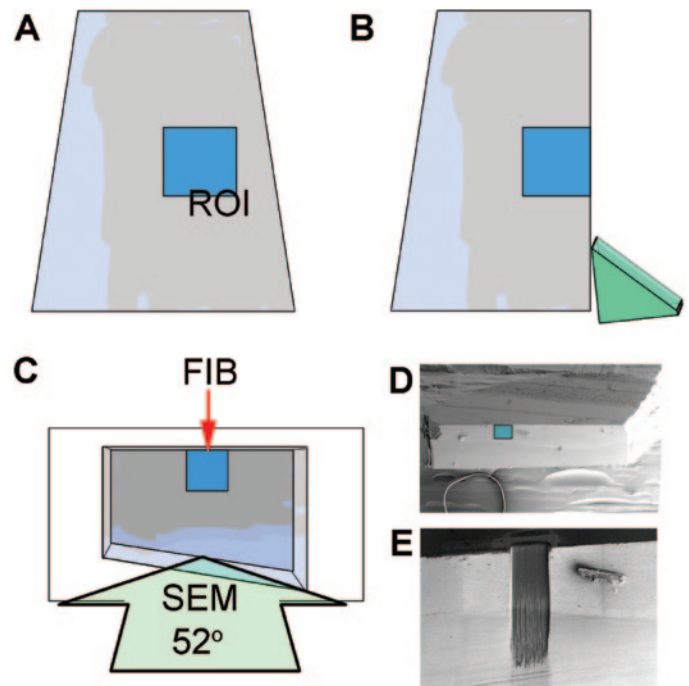

Figure 1: Trimming of embedded tissue in an ultramicrotome expedites FIB setup. The block is trimmed and mounted for conventional sectioning (A), and a region of interest (ROI, blue area) is identified by LM or TEM. Mounted sideways in the chuck, the block "edge" is then trimmed at 90º to the block face and cut with a glass knife (B). This places the ROI at a flat, perpendicular "top" edge from which the FIB mills (C). Panel (D) illustrates a large block face containing an entire mouse optic nerve (ROI overlaid in blue). After 300 slices were milled from that surface (E), the region of interest is obvious by SEM (E, higher magnification), facilitating rapid sample remounting and further scanning/slicing if required.
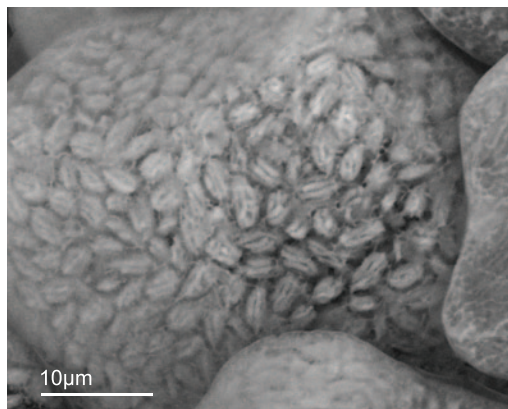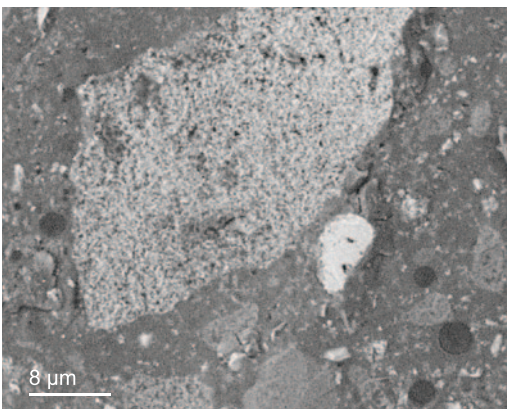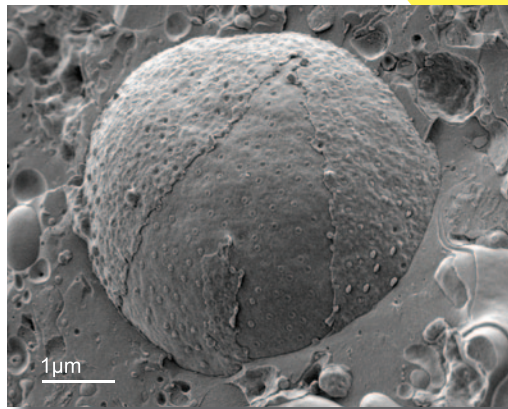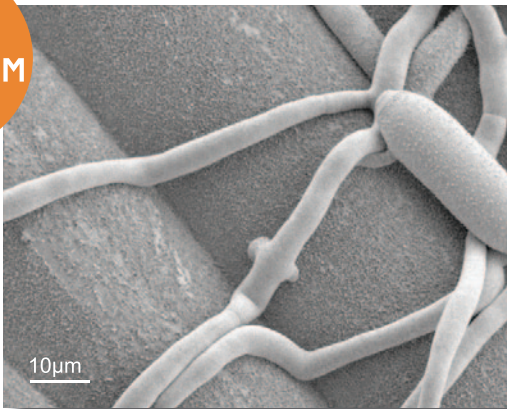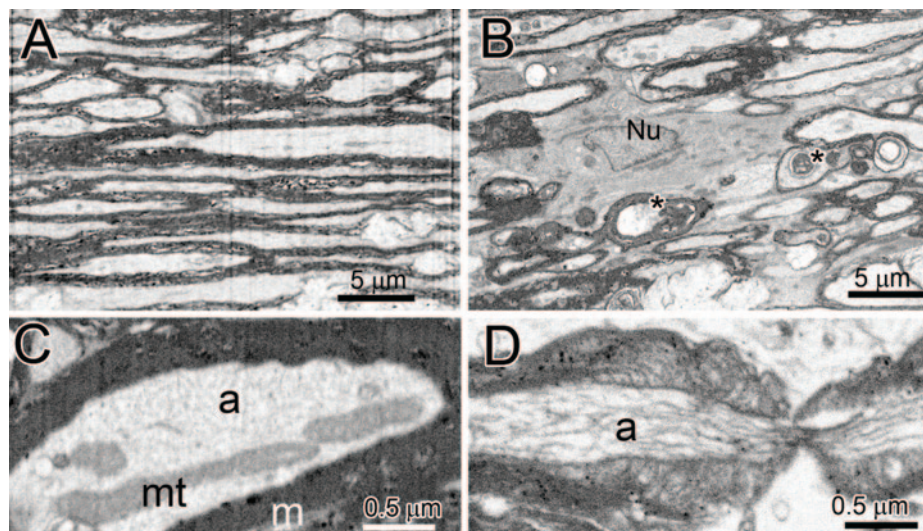
Figure 2: Optic nerves imaged using backscatter SEM after milling. In both wild-type control optic nerve (A) and nerve from Po-CNS mice (B), myelin and axons are well-contrasted and damaged myelin (B,*) is obvious. Cell nuclei (B, Nu) and other features are also clearly identified. In routine preparations (C), mitochondria (mt) were readily resolved in the axon (a). Addition of tannic acid treatment (D) enhanced contrast of microtubules, neurofilaments, and some paranodal structure. Myelin (C, m) appears black in these images, which are negative (inverted) images of the original backscatter data, so as to resemble conventional TEM images.

involves fixation of the tissue, infusion with contrast-generating heavy metal stains, then embedding in epoxy resin. In these experiments, nerves were obtained from 6-9 month old control (wild-type) mice and from P0-CNS mice, a model animal in which genetic alteration of myelin produces substantial axonal pathology in older mice [2]. Nerves were fixed by perfusion with 2.5% glutaraldehyde and 4% formaldehyde in phosphate buffer and then treated 1 hour in buffered 0.4% osmium tetroxide. They were then stained en bloc in ethanolic uranyl acetate, which is not routine in our TEM preparation, and then dehydrated and embedded in Durcupan® resin (Electron Microscopy Sciences) in a flat mold. We used Durcupan because it had been shown to be stable in FIB/SEM [1], but other EM embedding resins may work just as well. Tannic acid is well known as a contrast enhancement agent for cytoskeletal imaging by TEM. In some experiments, tissues were incubated in 1% tannic acid for 1 hour prior to the osmium tetroxide step to test whether this would also aid backscatter electron imaging.

Although regions of interest (ROIs) for serial imaging can be identified dynamically after the block is mounted in the FIB system, in practice it is much faster to locate ROIs and trim the block on an ultramicrotome. Embedding resin was trimmed from around the tissue using a razorblade, creating a small raised mesa with the tissue exposed at the plateau (Fig 1A and 1D). Using a glass knife in an ultramicrotome, the block was "faced off" by cutting 50-100 1-μm sections. The last section was stained for LM (toluidine blue) and an ROI was selected (Figure 1A). Retrimming for FIB analysis entailed positioning the ROI immediately adjacent to the upper surface when it is mounted in the FIB (Figure 1B and 1C). That surface was also cut with the glass knife at right angles to the block face (Figure 1B), with the block mounted sideways in the ultramicrotome

chuck. These steps greatly reduced the amount of preparative milling that was necessary. Blocks were then sputter-coated with 300 nm of palladium.

Imaging Optic Nerves

TEM-like images are generated using the FIB/SEM approach by imaging backscattered electrons from the heavy-metal-impregnated tissues in the block face. Tissues were examined in a Nova Nanolab-200 Dual Beam FIB system (FEI) at the Swagelok Center for Surface Analysis of Materials at Case Western Reserve University. The section cutting was performed with a 30-kV gallium ion beam and a current of 1 nA or 3 nA. The SEM imaging was carried out with a 2-kV electron beam in high-resolution mode (that is, Mode 2, monopole magnetic immersion lens mode) using the through-the-lens detector in backscattered electron mode (collection tube negatively biased) with an electron beam current of 21 pA [1]. The images were acquired in a high-resolution format of 2048-1768 pixels and scanning time of 0.1 msec per line [1]. Automated milling and imaging was accomplished using the Slice & View software that was purchased with that system. These settings are similar to those used by Knott et al. [1], except that they were adapted for our specific instrument (discussed below).

Our first experiment with optic nerves confirmed that, using the high-resolution mode (immersion lens, mode 2), quantifiable images of optic nerve axons could be obtained (Figure 2A). Samples with pathology, such as myelin whirls (Figure 2B, asterisks) characteristic of axonal degeneration, were easily distinguished from intact axons and myelin. At magnifications of 2.5-20 KX, myelin, axons, and the organelles within the axoplasm were all sufficiently well resolved to measure axonal diameter, identify nodal regions, and resolve mitochondria (Figure 2C) from each other and adjacent organelles. Accumulations of pathological organelles characteristic of P0-CNS mice were readily apparent. Optic nerves contain about 50% myelin, and we were concerned that underlying heavily stained myelin may mask structures above it, as reported by Knott et al. [1], but by using a 2-kV beam for imaging, this effect was not prominent. We chose a lower electron beam current than that used by Knot et al. [1] because this produced the resolution required for the experiments with our older generation HRSEM column. Lower current, however, resulted in less favorable signal-to-noise ratio conditions and more work correcting the focus and astigmation.

The addition of a tannic acid preparative step before tissue osmification greatly enhanced the contrast of axonal mitochondria and microtubules (Figure 2D) and provided greater ultrastructural detail within regions of the myelin

such as paranodal loops. Some penalty was incurred, as many plasma membranes were less clearly contrasted and some punctate artifact was observed within mitochondria and myelin. These are a familiar trade-offs in using tannic acid for TEM. For purposes of imaging axons, mitochondria, and their cytoskeletal components, addition of tannic acid in the protocol made a large positive difference.

Serial "Slice and Scan" FIB Imaging

Because optic nerve axons have important structures at different size scales, several different imaging conditions were tried. We tested magnifications between 2.5 KX and 20 KX, imaging of regions between 50-200 mm wide, slice thicknesses between 30-100 nm, milling depths (that is, distance milled down the block face) from 10-40 mm, and numbers of slices from 25-300. The average imaging time was 9 minutes per milling/imaging cycle, most of which was SEM image acquisition time. Thus imaging took between 4-50 hours, plus typically 4 hours of pre-milling and alignment. For most of the milling/imaging cycles, the system ran unattended. On occasions during long acquisitions, we checked for excessive drift and stability and confirmed that software corrections were effective for focal that had changed due to surface milling.

For routine imaging of optic nerves, we found that a magnification of 5-10 KX worked well. At 7.5 KX, viewing fields covering 35 mm of optic nerve were generated (Figure 3). Series of 25-300 sections were generated, which allowed analysis of 20-100 individual axons (Figure 3). Slice thicknesses of 40 nm allowed organelles to be followed within optic nerve axons without large changes between slices that made interpretation difficult. Axons, mitochondria, and finer structures such as smooth endoplasmic reticulum could be readily traced between slices and their 3D structures reconstructed and displayed in a variety of space-filling depictions (Figure 4A).

Raw datasets required software post-processing in order to be easily interpretable. The SEM images are taken at an angle of (52°) to the block face (Figure 1C) and to compensate, images needed to be scaled vertically by 1.3x. Although this may be corrected during acquisition by the microscope software, that would result in a smaller field of view. Minor image drifting was also observed, involving translation of images vertically and horizontally of up to ~5 μm within the stack. Correction for this requires relatively simple image registration, and there are multiple software options available. We used NIH ImageJ software [3] for these tasks, using the "FIJI" image processing implementation including the SIFT image registration plugin [4] implemented in a small macro, which stepped through the stack and aligned each slice to the preceding slice. We used ImageJ because it is relatively simple to use, designed to process image stacks, and as open source software, licensing is not an issue when setting up multiple workstations. Once registered, axons were easily followed between slices and could also be re-sliced to view the stack from orientations orthogonal to the plane of section (for example, Figure 3, side view). The tracing of axons and mitochondria and 3D reconstructions of these structures (Figures 4A-4C) was undertaken using another open
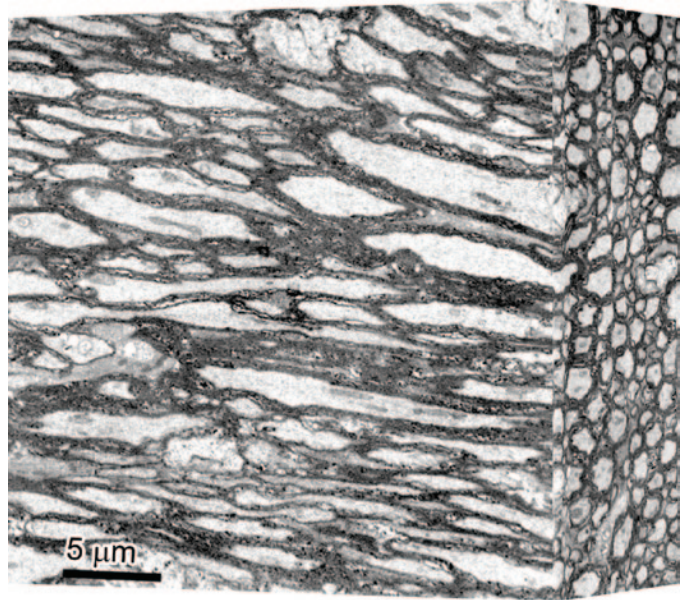

Figure 3: A stack of 242 slices from a normal mouse optic nerve reconstructed in perspective view. Tissue was imaged at a magnification of 7.5 KX with a 40-nm slice thickness. Approximately 100 axons are included in this stack.

source program, Reconstruct [5], working with TIFF images exported from ImageJ. Axons were easily segmented using the automated "wildfire" tracing features, but mitochondial tracing entailed a combination of automatic and manual tracing. Final 3D models were generated with Blender software [6] and assembled with other views in Adobe Photoshop. Tracing sections remains a significant bottleneck, and more sophisticated image analysis approaches may greatly enhance throughput.

Minor artifacts and limitations of the FIB technique were evident in serial sectioning experiments. As mentioned by others [1,7], streaking or "curtaining" was evident as vertical lines on some images, which is a result of uneven milling. Several factors reduced this effect. Platinum deposition on the "top" surface of the block had a major impact, however, adequate milling of the block face prior to imaging and using small milling steps during pre-milling and imaging cycles were also important. Curtaining was never entirely eliminated, but it was intermittent in a sequence and usually could be worked around. An allied problem was inconsistency in slice thickness, a problem previously noted [1]. Changes in slice thickness were immediately obvious when stacks were viewed as a movie or when they were re-sliced and circular profiles of the axons or organelles were examined for eccentricity. Variation tended to affect contiguous groups of sections, and judicious stack curation was possible using spherical or circular organelles as internal reference indices. In practice, neither curtaining nor variations in slice thickness had a significant impact on optic nerve analysis and were analogous to problems of section folding, knife and chatter marks, and missing/damaged sections that can plague conventional TEM serial sectioning. Further, the sheer number of sections obtained using the FIB approach means that large numbers of axons are sampled even
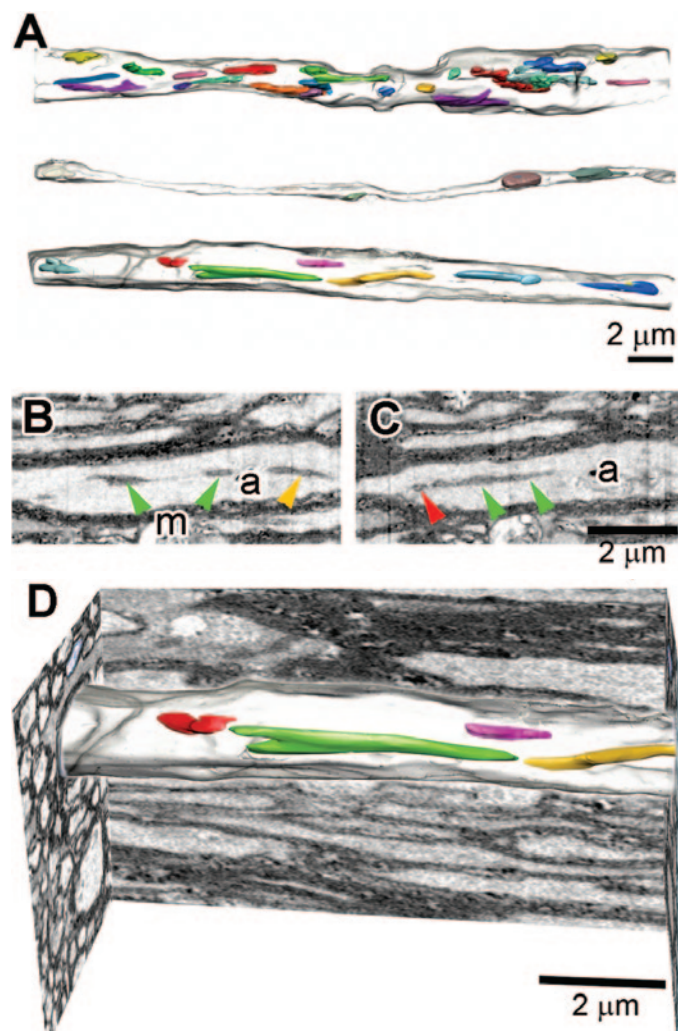
Figure 4: Three-dimensional reconstructions of mitochondria in optic nerve axons. Distributions and sizes of 38 individual mitochondria in three representative axons are illustrated in (A). Serial analysis of optic nerve allows discrimination between multiple short mitochondria and long, potentially branching mitochondria. In two single slices (B, C), several mitochondrial profiles are present (arrowheads). In 3D reconstructions (C), individual mitochondria are resolved and the bifurcation of the "green" mitochondrion is readily apparent.

if occasional regions of the stack or section need to be excluded.

The dual FIB is in fact two microscopes in one system and this means there are drifts related to the SEM imaging as well as ion beam slicing. Drift in the SEM images was addressed post-acquisition as described above. Ion beam drift can be a critical issue because large variations will result in destruction of the ROI. Some new dual beam systems offer software solutions that allow monitoring and correction of ion beam drift during runs using fiducial marks. An alternative option for dedicated users who wish to tailor the working conditions to their own needs is to write their own script to control the microscope slicing and imaging. This could give the user freedom to perform experiments where one may acquire data using various settings within one experiment. Using our software version we were able to manually change the magnification within one experiment and effectively carry out two experiments, one after the other. By having direct control one may, for example,

capture two images at different magnification for every slice and have two data sets for each slice. Alternatively, one may capture higher magnification images at small intervals and capture a larger field of view every few slices.

Practical Considerations

A significant practical factor in developing dual beam applications for biological analysis is the importance of collaboration. Close collaboration is almost obligatory, as sample preparation and image interpretation require familiarity with biological TEM and, in this case, a neuroscience background. FIB milling and SEM expertise are likely to come from materials scientists or engineers. Digital imaging skills are also required, and speeding up analysis in the future is going to require collaborations with experts in image processing.

Choices of magnification and slice thickness required the greatest consideration. Conflicting goals of resolving organelles while sampling the greatest possible number of axons requires compromises, and choices must ultimately be dictated by the question being asked of the dataset. A clear plan of what structures need to be critically resolved in each set is thus important. Myelin thickness and internodal length can be measured at 2 KX-5 KX with 100-nm milling steps to maximize axons counted. Mitochondrial distributions required 5-10 KX with 40-nm slices to trace them reliably (Figure 4), whereas microtubule orientation required finer steps and higher magnification. In contrast, deciding how many slices to collect was usually simple because it is determined by the availability of the FIB during off-peak hours (that is, over the weekend).

Conclusions and Perspectives

The dual beam slice and view approach offers an unparallel tool for analyzing and quantifying axonal structure, myelin organization, and organelle distributions in optic nerve axons and other CNS tissues. Enhancing contrast by adding tannic acid pretreatment will be worthwhile for many applications. Time and financial costs necessitate serious experimental planning, and *a priori* TEM or LM examination of samples should be used to confirm that each sample is suitable. Nevertheless, this "high throughput" approach to serial-section electron microscopy of optic nerves has opened up new territory and questions that were not amenable to existing EM or confocal approaches [8].

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