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Three Dimensional Ultrastructural Analysis of CNS Axons Using Serial Ion-Abrasion Scanning Electron Microscopy (SIA-SEM)

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Axonal dysfunction and degeneration underlie many central nervous system (CNS) diseases, including those affecting the myelin sheath in individuals with multiple sclerosis [1]. Studies of the pathogenesis of CNS axonal damage in human tissues and experimental animal models are often impeded by the small size of most CNS axons (0.5-2 μm). Myelinated axons in CNS white matter tracts are densely packed with little extracellular space between them. Key axonal organelles, including mitochondria and microtubules, are at or beyond light resolution limits, which makes it difficult to differentiate them from one another using light and confocal microscopy techniques. TEM provides high resolution in single slices but obtaining three dimensional (3D) data, such as organelle lengths, shape information and distribution along each axon is not straightforward.

Recent advances in serial ion–abrasion SEM (SIA-SEM) imaging have provided new options for generating serial images with 10 nm resolution or better. In dual beam focused ion beam systems, the surface area of the region of interest is milled with a focused Ga ion beam and then imaged with an SEM using backscattered electrons [2-4]. This method offers several advantages for imaging CNS axons: data collection is automated, data sets allow reconstruction of axons and their organelles in three dimensions, and the tissue can be prescanned to locate regions of interest, such as areas in which axons are in longitudinal orientation. Another advantage is availability of dual beam microscopes, which are common in nanofabrication and material science labs. In previous work, we have investigated some technical issues in applying a FIB approach to examination of CNS white matter axons [5]. In this paper we focus on the practical issues of obtaining and using this approach as a routine analysis method in the lab.

Samples of cerebellar white matter were obtained from three wild-type rats aged 30d old, an age at which myelination of CNS axons is largely complete, and optic nerves from two 6 month wild type optic nerves. Animals were perfusion fixed with 2.5% glutaraldehyde and 4% paraformaldehyde, then treated with buffered 0.4% osmium tetroxide, washed and stained in ethanolic uranyl acetate. Samples were dehydrated and embedded in Durcupan resin as previously described [2, 5]. To enhance cytoskeletal and mitochondrial contrast at low magnification, samples were also tannic acid treated prior to osmification [5]. Blocks were trimmed, sputter coated with 30nm palladium, and examined in a Nova-200 Nanolab Dual Beam FIB, (FEI) system at the Swagelok Center for Surface Analysis of Materials at Case Western Reserve University.

Milling of the tissue block face was performed with a 1nA ion beam current and imaged in high-resolution mode (immersion lens) using the through lens detector in backscattered electron mode. The images were acquired in a high resolution format of 2048-1768 pixels and scanning time of

0.1msec per line. Images were viewed, registered, corrected for aspect ratio, and derivative stacks generated using the FIJI implementation of Image J software [6].

For routine analysis, series of 200-400 images at ~40 nm steps were obtained at x7500, which took 36-60 hrs of milling and scanning. At this magnification, viewing fields covered 35 μm wide by 45 μm high and 8-16 μm deep and included ~50-100 axons that could be traced for 20-50 μm in three dimensions. Organelles such as mitochondria were readily visible at this magnification, although higher magnification images were required to identify mitochondrial cristae and microtubule associations. Long acquisition times result in some drift (5-10μm) in image registration between slices, but this was easily corrected using registration software. Some less-pronounced drift within the images due to the long acquisition time was also occasionally observed which could not be easily corrected and may constrain precision of volumetric data. Disparity in the slice thickness was also a factor, and was compensated for during volume tracing using to spherical structures to calibrate section thickness, as described by Fiala and Harris [7].

Three dimensional distributions of mitochondria in axons were determined using a combination of manual and semi-automated (wildfire) tracing using Reconstruct software [8]. Although manual tracing is labor intensive, at magnifications that optimized tissue sampling (x7.5K), the similar electron densities of mitochondria and adjacent myelin made fully automated approaches difficult. Future availability of higher pixel density images and/or image stitching will permit acquisition of wider fields of view at higher magnifications and facilitate automated segmentation. While Reconstruct software provides volume and distance data, we found it convenient to export the data and analyze it with simple, purpose-written software to calculate volumes and geometrical relationships and collate axons and their mitochondria. This permitted rapid recalculation as new axons and features were included, and provided customized output files for statistical analysis. Data were validated by direct measurements from 3D data sets.

Data obtained from 45 cerebellar axons and 18 optic nerve axons included 744 mitochondria and indicated that in both young rats and old mice, mitochondria present in the nodal regions of the axon were significantly smaller and more spherical than those located internodally. Internodal mitochondria had a characteristic clustered distribution that resembled the arrangement of stationary mitochondria observed in parallel studies of mitochondrial dynamics. Preliminary studies of CNS from mice with mutated myelin proteins suggests that changes in distribution and shape assessed by 3D SIA-SEM may be sensitive early markers for axonal pathology.

References:

[1] Trapp B.D. and Nave. K-H. *Annual Review of Neuroscience,* (2008), 31: 247
[2] Knott et al, *The Journal of Neuroscience* (2008), 28(12):2959.
[3] Bennett et al., *PLoS Pathog* (2009), 5(9): e1000591.
[4] Hekking et al*., J Microscopy*. (2009), 235(3):336-47.
[5] Kidd et al., *Micoscopy Today.* (2010), 18(1):18-22
[6] FIJI implementation of Image J http://pacific.mpi-cbg.de/wiki/index.php/Main_Page
[7] Fiala J.C. and Harris K.M.. *J Microscopy.* (2001), 202:468-72
[8] Fiala J.C. *Journal of Microscopy* (2005), 218:52-61.

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