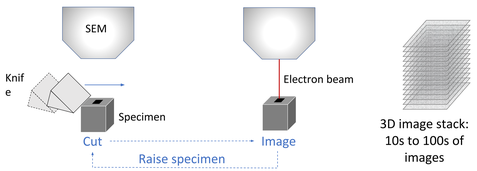
Serial Block-Face SEM of Brain Tissue Using Rapid Automated Preparation

Emily K Benson,[1] Grahame J Kidd,[1] Jay M Campbell,[2] Steven L Goodman[2,3]

1. 3DEM Ultrastructural Imaging & Computation Core, Lerner Research Institute, Cleveland Clinic, Cleveland, OH USA
2. University of Wisconsin, Depts. Neurological Surgery, Pathobiological Science & Neuroscience, Madison, WI, USA
3. Microscopy Innovations, LLC, Marshfield, WI, USA

Introduction

Serial block face scanning electron microscopy (SBEM) provides high-resolution 3D imaging of neurological structures. 3D SBEM images are acquired by obtaining 100s of sequential image "slices" of a specimen "block" at increasing depths.



While SBEM image acquisition, and image analysis, are extensively automated, SBEM sample preparation is intensely manual. Tissues are chemically processed, similarly to TEM specimens, but with additional toxic and reactive steps of extended duration. The preparation process typically requires a work week of manual processing.

Sample staining and embedding are largely diffusion limited reactions. Reduced duration preparation is possible using automated systems such as the ASP-1000™ with mPrep/s capsules, which accelerates diffusion into specimens with gentle alternating fluid flow every few seconds. The present study was undertaken to test whether samples obtained are comparable qualitatively and quantitatively with those from manual preparation.

Experimental

Tissues were excised from perfusion-fixed rats, and stored at 5°C in buffered fix until subsequent preparation (Fig. 1). For ASP prep, ~1-3 mm tissue pieces were entrapped in mPrep/s capsules and the capsules were loaded onto the ASP. Reagents were dispensed into microwell trays and sealed with pierceable covers (Fig. 1). The ASP executed the SBEM protocol (Table 1) by aspirating successive reagents into capsules for the programmed time. Agitation was provided by gentle flow reversals through the capsules every 2-5 seconds. Epoxy infiltrated specimens in capsules were removed from the ASP and cured overnight at 60°C. Manually processed (control) specimens were prepared in vials (Table 1).

	Manual		Automated	
Reagent	**Exchanges**	**Time (min)**	**Exchanges**	**Time (min)**
Karnovsky fix	perfuse	Store 5C	perfuse	Store 5C
Buffer	6	30	30/30/30*	3
Tannic acid (some)	1	15	450	15
OsO4 - KFeCN	1	180	1800	60
Water	5	25	90/90/90/90/90	15
1% TCH	1	60	1800	60
Water	5	25	90	3
2% OsO4	1	180	900	30
Water	5	25	90/90/90/90/90	15
2% Uranyl Acetate	1	1,200	1800	60
Water	5	25	90/90/90	9
Lead Aspartate	1	40	900	30
Water	5	25	45/45/45	9
Graded ethanols	15	105	630	53
Acetone	2	30	270/270	9
Epoxy-acetones	2	600	900	30
100% epoxy	1	90	120/120/120	30
Transfer tissue to molds	1	45	NA	0
Resin cure 60C	Into oven	2 days	Into oven	Overnight
Time : Effort	**4 elapsed days : 2 days work**		**1 elapsed day : 1 hour work**	

Table 1: Protocol, reagent exchanges and incubation times for reagent exchanges. *30/30/30 indicates 30 exchanges in 3 different reagent wells.

Samples were imaged using a ThermoFisher/FEI Teneo VolumeScope Serial Blockface Imaging system equipped with a fresh Diatome Diamond Knife, at 2.0 kV, 0.1 nA under high vacuum using the T1 detector.

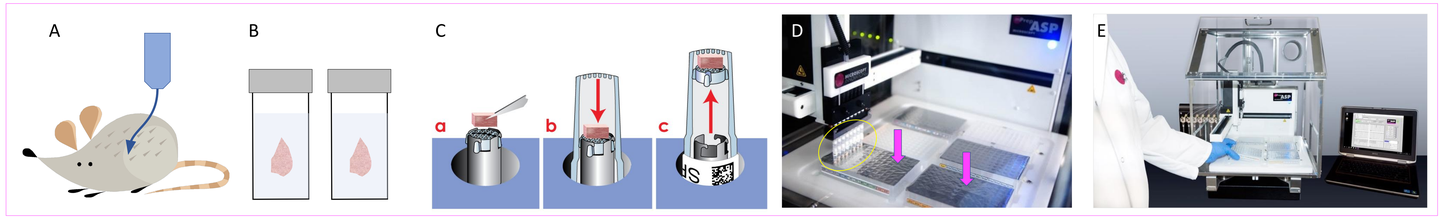


Figure 1: A) Rats were perfused with Karnovsky's fixative. B) Tissues excised & stored in fix at 5°C. C) Tissue oriented and entrapped in mPrep/s™ capsules using an mPrep/s Workstation. D) Eight specimens in capsules (circled) on ASP-1000™. Reagents in sealed microwell plates (arrows). E) ASP-1000 with fume enclosure and computer control unit (right). Note reagent microwell plate is being placed on reagent deck.

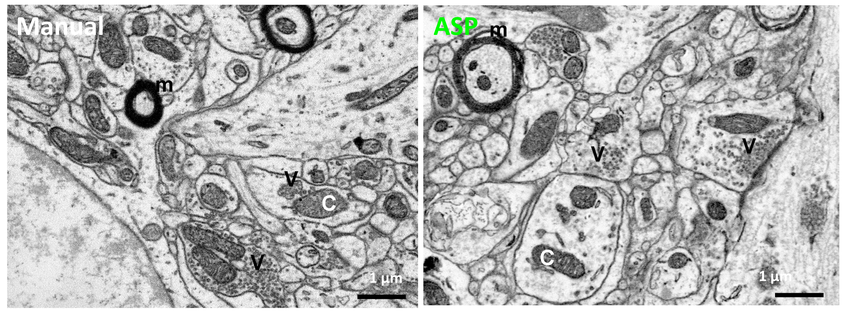


Figure 2: Manual & ASP prepared cortex, single EM plane views. Myelin (m), synaptic vesicles (v), mitochondrial cristae (c).

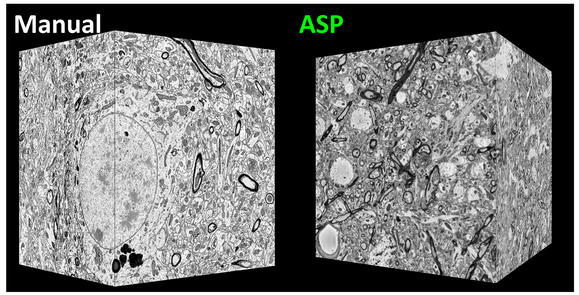


Figure 3. Perspective projections cortex prepared manually and with ASP. ~60 x 60 x 20 µm deep, from 350 70 nm thick slices. Images acquired in 25 hr each.

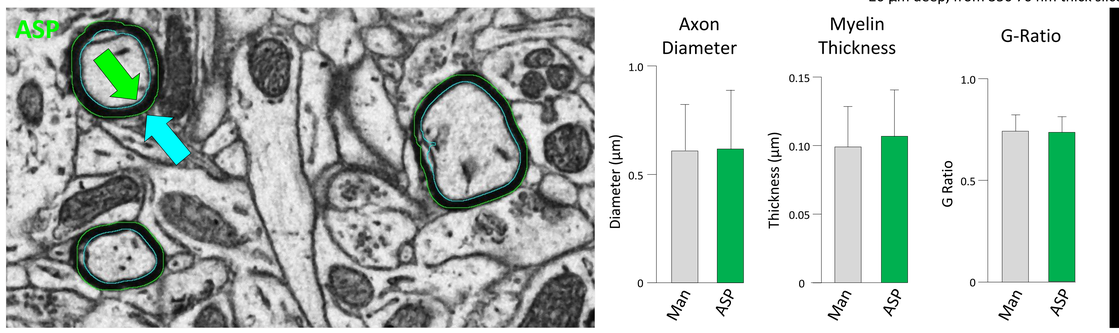


Figure 4: Automated analysis of axon diameter (cyan), myelin thickness (green) & G-ratio = axon diameter/myelin outside diameter; 4,894 axons measured: mean ± SD.

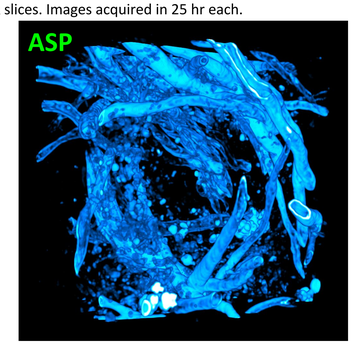


Figure 5. Segmented myelin.

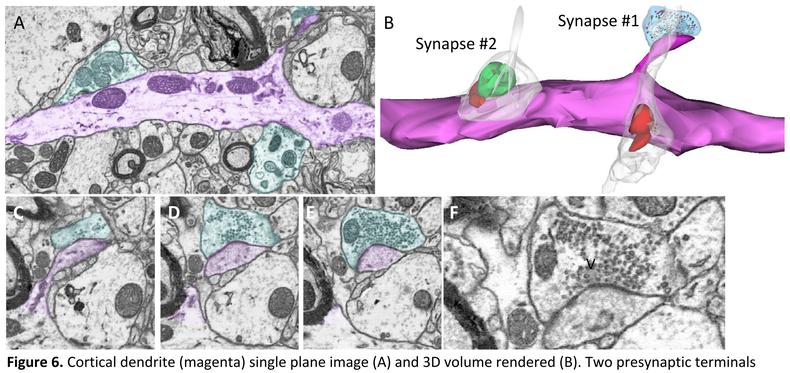


Figure 6. Cortical dendrite (magenta) single plane image (A) and 3D volume rendered (B). Two presynaptic terminals highlighted in cyan & cyan/white show internal mitochondria and vesicles (red, green) detailed below: Synapse #1 shown in slices (C-E) and high resolution (F) show vesicle (V) distribution and structure.

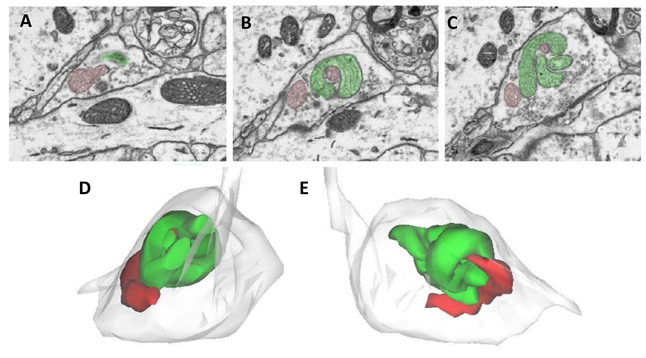


Figure 7. Two mitochondria in Fig 6, synapse 2 (A-C), colored green & red, exhibit convoluted structure that is fully appreciated in 3D volume reconstruction (D), shown with 90° rotation (E).

Results and Discussion

ASP-1000 tissues were prepared in less than 8 hours, followed by overnight resin curing. Operator effort required ~45 minutes to load specimens and reagents prior to processing, and ~15 minutes to transfer resin infiltrated specimens to an oven for polymerization and for cleanup. Manual processing required 13 hours of hands-on reagent exchanges over several days, plus 2-3 overnight steps.

SBEM quality was comparable for manual and ASP-prepared brain (Figs. 2-4). Both provided high-quality specimens that exhibited good sectioning properties using stock Diatome diamond knives. Myelin was well-stained (Fig. 2-5), indicating complete reagent penetration, since lipid-rich myelin membranes are both a dense target of metal staining and a barrier to diffusion. Synaptic vesicle clarity and discrimination of mitochondrial cristae against the dark mitochondrial matrix were also comparable (Fig. 2-3). Volume rendering and segmentation of ASP-prepared cortex provided detailed 3D information on myelinated axon locations (Fig. 5) and synaptic architecture, including vesicles and mitochondrial structures (Fig. 6, 7).

Quantitatively, axon and myelin dimensions were the same for manual and ASP prepared tissue (Fig. 4), indicating that rapid preparation has not exacerbated shrinkage.

Summary

Automated staining and embedding using ASP-1000™ demonstrated several benefits for SBEM (and FIB-SEM) workflow:

- Automated specimen prep in 1 day, instead of a week
- Operator effort reduced to 1 hour, instead of 2 days
- Reduced handling and exposure to toxic reagents
- No need to hold samples for large-batch manual staining.
- Provides automated consistency and reproducibility

The present study indicated that ASP produced samples comparable to manual preparations for:

- Detection thresholds for small structures (cristae and vesicles)
- Quantitative measurements of micrometer-sized structures.

Future potential

Protocols are readily modified to alter stains, perform en bloc immuno-gold labeling [3], or change other parameters [2].

While SBEM preparation was only demonstrated herein, the ASP may also prepare tissue for focused ion beam and array tomography, since preparative methods are similar. The ASP could also provide SBEM immuno labeling, as demonstrated for TEM [3].

References

1. TJ Deerinck, et al, NCMIR methods for 3D EM: A new protocol for preparation of biological specimens for serial block face scanning electron microscopy. Microscopy online (2010), 6. http://ncmir.ucsd.edu/sbfsem-protocol.pdf.
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