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

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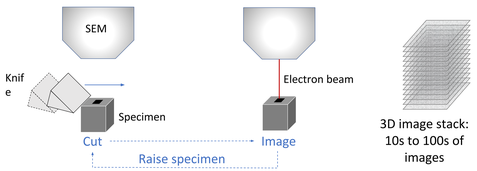
Introduction

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.

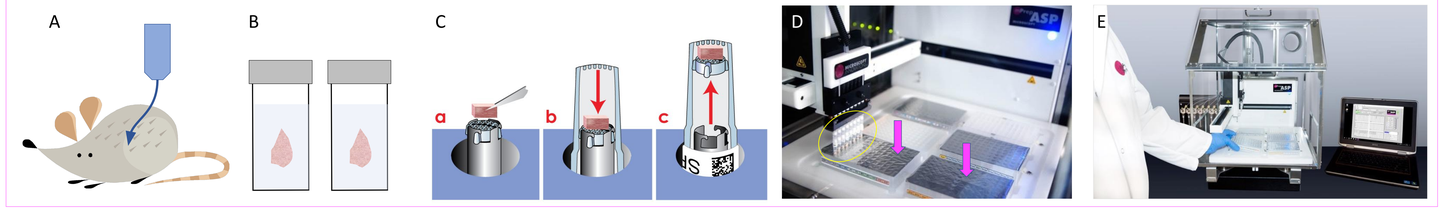


Figure 1: A) Rats were perfused with Karnovsky's fixative. B) Tissues excised & stored in fix at 5°C. C) Tissue oriented and entrapped in mPrep/s™ capsules using an mPrep/s Workstation. D) Eight specimens in capsules (circled) on ASP-1000™. Reagents in sealed microwell plates (arrows). E) ASP-1000 with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

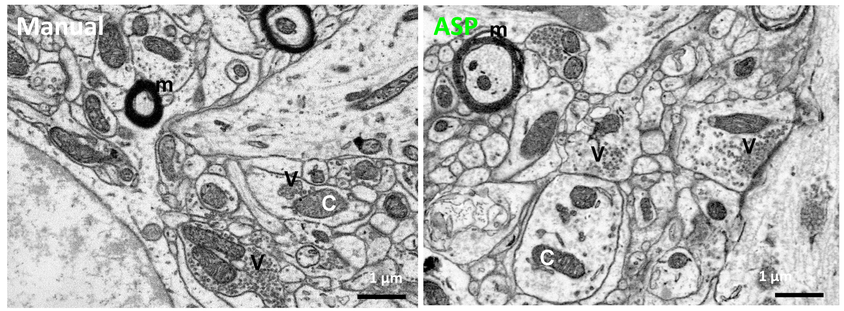


Figure 2: Manual & ASP prepared cortex, single EM plane views. Myelin (m), synaptic vesicles (v), mitochondrial cristae (c).

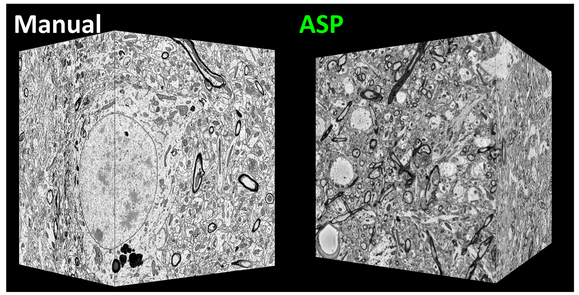


Figure 3. Perspective projections cortex prepared manually and with ASP. ~60 x 60 x 20 µm deep, from 350 70 nm thick slices. Images acquired in 25 hr each.

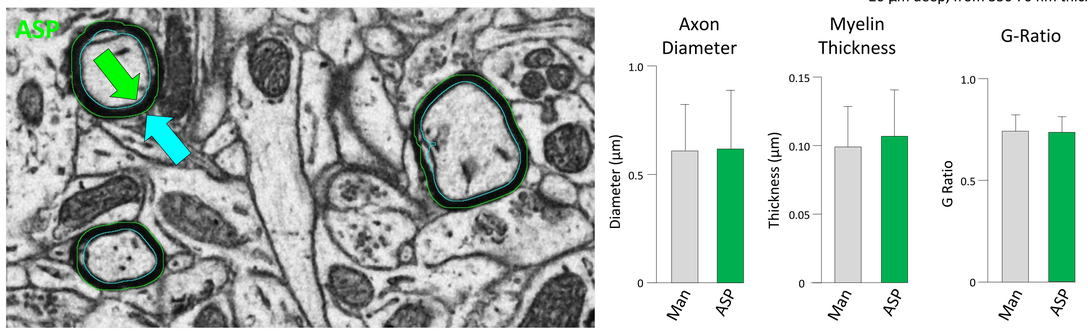


Figure 4: Automated analysis of axon diameter (cyan), myelin thickness (green) & G-ratio = axon diameter/myelin outside diameter; 4,894 axons measured: mean ± SD.

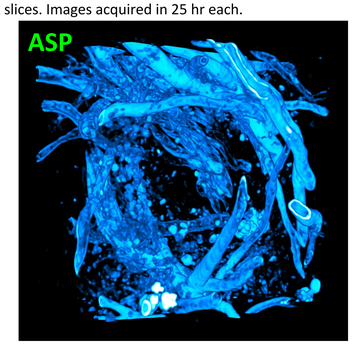


Figure 5. Segmented myelin.

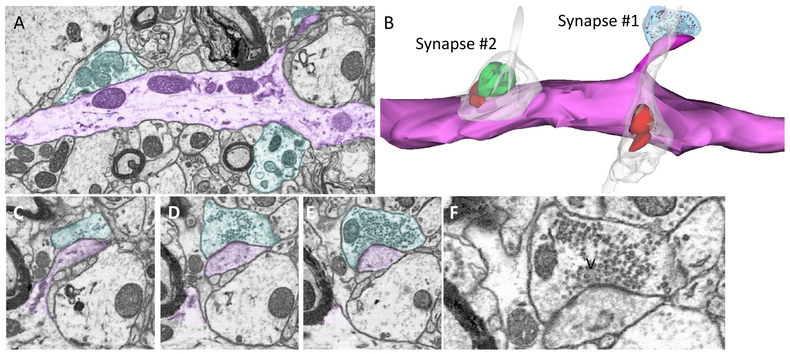


Figure 6. Cortical dendrite (magenta) single plane image (A) and 3D volume rendered (B). Two presynaptic terminals highlighted in cyan & cyan/white show internal mitochondria and vesicles (red, green) detailed below: Synapse #1 shown in slices (C-E) and high resolution (F) show vesicle (V) distribution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

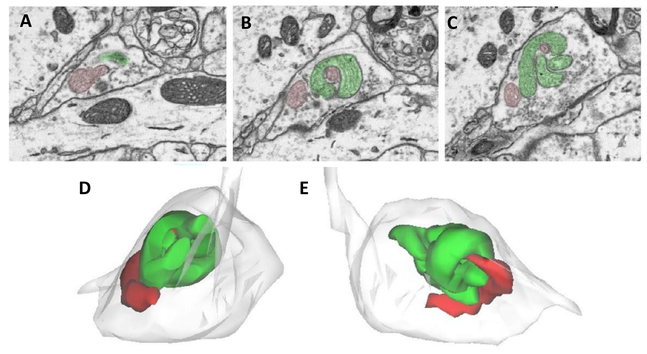


Figure 7. Two mitochondria in Fig 6, synapse 2 (A-C), colored green & red, exhibit convoluted structure that is fully appreciated in 3D volume reconstruction (D), shown with 90° rotation (E).

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.
2. TE Strader, et al. Automated rapid preparation of tissue specimens for TEM pathology. Microsc. Microanal 24 (2018), 1284
3. P Marques, J Strong, et al, Optimization of Automated Immuno EM for Both Pre- and Post-Embedding Labeling. Microsc. Microanal 24 (2018), 1300.

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Serial block-face electron microscopy (SBEM) provides powerful 3D insight into neurological structures for mapping and quantification. While image acquisition is now largely computer controlled, SBEM sample preparation requires tedious circa week-long manual exchanges of toxic reagents, that are similar to but even more extensive and laborious than the chemical sample preparation required for transmission electron microscopy (TEM).

We report herein an SBEM protocol for neurological tissue where all reagent exchanges are automatically performed in one day using the programmable mPrep ASP-1000 Automated Specimen Processor (ASP). The ASP enables high-speed preparation of biological tissues with rapid and repeated fluid exchanges that accelerate reagent diffusion into specimens. This has been previously demonstrated for TEM [1]. For SBEM, a 4 to 5 day manual preparative protocol [2] was reduced to 7.5 hours of automated processing, followed by resin curing.

Adult rats were perfusion-fixed with cacodylate-buffered glutaraldehyde-paraformaldehyde. For ASP preparation, cortex specimens (1-3 mm) were oriented in mPrep/s capsules, loaded onto the ASP, and all preparative reagents were aliquoted into microwell plates on the ASP stage. The ASP executed the protocol by aspirating successive reagents into each capsule for the programmed time (Fig. 1, Table 1) with agitation provided by gentle flow reversals every few seconds. Epoxy infiltrated specimens in the capsules were then removed from the ASP and cured overnight at 60C. Manual preparation was performed in vials and cured in flat molds [2]. A ThermoFisher Teneo VolumeScope imaged blocks at 2.0 kV, 0.1 nA under high vacuum with the T1 BSE detector. Imaged volumes were ~60 x 60 um by 20 um from 70 nm sections.

Specimens prepared with the 7.5 hour ASP protocol (plus resin curing) and the 4-day manual protocol were compared from the same animal. Myelin staining in both indicated good reagent penetration since lipid-rich myelin is a dense target for metal staining and a barrier to diffusion. Synaptic vesicle clarity and discrimination of mitochondrial cristae against the dark mitochondrial matrix were also comparable. Neither ASP or manually-prepared specimens exhibited deficits characteristic of poor staining, dehydration, or infiltration (Fig. 1).

SBEM can reveal complex 3D neural architectures that are not apparent from single plane images. Figure 2 shows a cortical dendrite in a single plane image (not unlike a TEM section) and a 3D volume rendered reconstruction from the SBEM data, which reveals complex pre-synaptic features that are not apparent in single plane images. With ASP-enabled automation of SBEM preparation, the ability to image such complex 3D neurological (and other tissue) structures becomes more easily achieved with less effort and greater reproducibility. Additionally, since the ASP reduces reagent handling, and as it vents into or is contained in a fume hood, experimenter exposure to noxious reagents may also be reduced.

Reagent	Manual Time (min)	ASP-1000 Time (min)
Karnovsky fix	Store 5C	Store 5C
Buffer	30	3
Tannic acid (some)	15	15
OsO4 - KFeCN	180	60
Water	25	15
1% TCH	60	60
Water	25	3
2% OsO4	180	30
Water	25	15
2% Uranyl Acetate	1,200	60
Water	25	9
Lead Aspartate	40	30
Water	25	9
Graded ethanols	105	53
Acetone	30	9
Epoxy-acetones	600	30
100% epoxy	90	30
Transfer tissue into molds	45	0
Resin cure 60C	2 days	Overnight
Protocol time	**4 days**	**1 day**
Hands-on labor time	**2 days**	**1 hr**

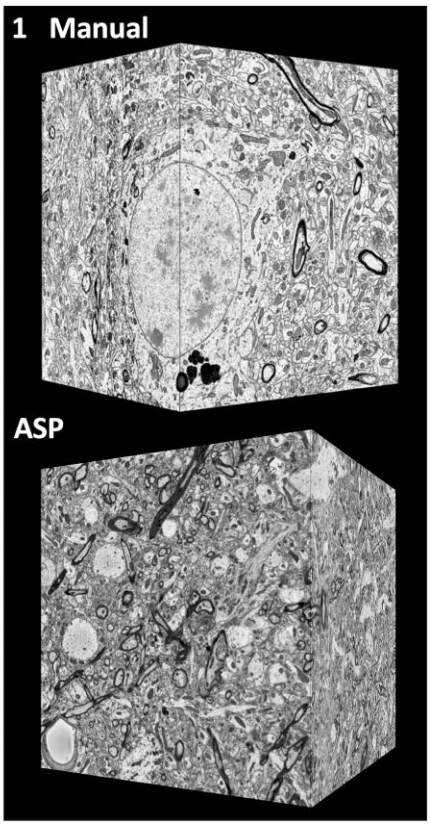

Figure 1. Table 1: Reagent protocol, times, and hands-on labor effort for manual and ASP preparation. Figure 1: Perspective projections of cortex prepared manually (top) and with ASP (bottom). ~60 x 60 x 20 μm deep, from 350 70 nm thick slices. Images acquired in 25 hr each.

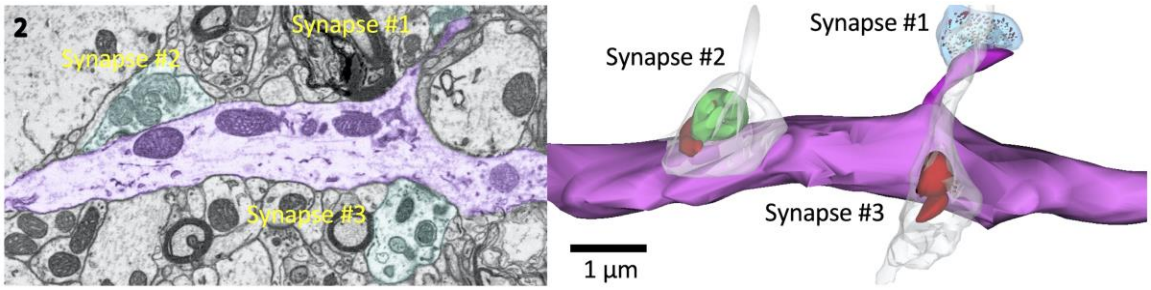

Figure 2. Cortical dendrite (magenta) single plane image (left) with 3 synapses and 3D volume rendered image (right). 3D rendering show complex architecture including synaptic vesicles in Synapse #1, and mitochondria in Synapses #2 and #3.

References

[1] Strader TE, et al, Microsc. Microanal. 24 (2018) 1284.
[2] Deerinck, TJ, et al, Microscopy, (2010), 6.