doi:10.1017/S1431927619006640
Microsc. Microanal. 25 (Suppl 2), 2019

Rapid Automated Preparation for Serial Block Face Scanning Electron Microscopy

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

[1.] Microscopy Innovations LLC, Marshfield, WI, USA.
[2.] Renovo Neural Inc., Cleveland, OH, USA.
[3.] University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI, USA.
*Corresponding author: Steven.Goodman@MicroscopyInnovations.com

By providing automated acquisition of large volume electron microscope image stacks, serial block-face electron microscopy (SBEM) creates exciting new possibilities for life science discovery. While many repetitive aspects of microscopy are now computer-controlled, sample preparation remains a hurdle. SBEM sample preparation uses chemical fixation, heavy metal staining, and resin embedding protocols based on those developed for TEM, plus additional toxic and reactive steps. While TEM preparation typically requires 1-2 days, nearly week-long SBEM protocols are used to provide the heavy metal staining and conductivity required for high-contrast back-scattered SEM imaging at 1-3 KeV.

We report herein an SBEM protocol for neurological tissue where all reagent exchanges were automatically performed in one working day with the 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, which can prepare tissues for TEM as rapidly as 1-3 hours [1]. For SBEM, a 13-hour hands-on manual protocol with 3-4 overnight treatments was accelerated into a 7.5-hour automated protocol, with only one overnight for resin curing.

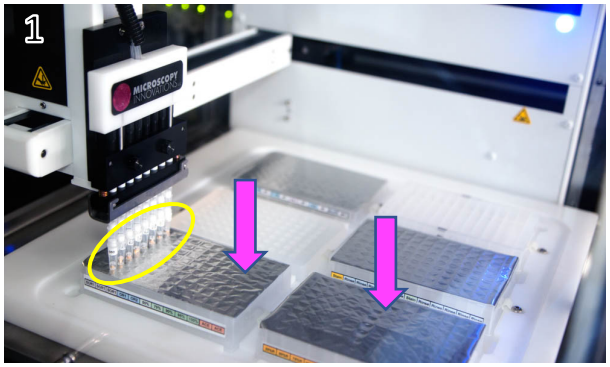
Brain was excised from a perfusion-fixed 12-month old rat, then immersed and refrigerated in cacodylate-buffered glutaraldehyde-paraformaldehyde until subsequent preparation. Cortex and corpus collosum specimens (1-3 mm) were loaded into labeled mPrep/s capsules. Capsules were mounted onto the ASP and reagents were dispensed into microwell trays with light sensitive and reactive reagents tightly covered using microplate sealing foil (Fig. 1). The ASP executed the protocol (Fig. 2) by aspirating successive reagents into each capsule for the programmed time, breaching foil seals as needed. Agitation was provided by gentle flow reversals through the capsules every few seconds. Epoxy infiltrated specimens in capsules were removed from the ASP and cured overnight at 60C. An FEI Teneo VolumeScope imaged blocks at 2.0 kV, 0.1 nA under high vacuum using the T1 detector. Images ~60 x 60 um and 20 um deep (350 slices with 70nm cuts) were acquired in 25 hr runs. Figure 3 is a perspective projection SBEM image of a sample of cortex prepared with the ASP.

To compare preparation quality, samples from the same animal were prepared manually (Fig. 4a) and with the ASP (Fig. 4b). The evenness of myelin staining in both indicate good reagent penetration since lipid-rich myelin membranes are both a dense target of metal staining and a barrier to diffusion. Synaptic vesicle clarity (Figs. 4a-b) and discrimination of mitochondrial cristae against the dark mitochondrial matrix (Figs. 4a-b), were also comparable. Neither the ASP or the manually-prepared specimens exhibited common SBEM problems including brittle, cracking, or shrinking blocks, or blocks that produce shards during cutting. Overall, the ASP produced comparable preparations in much less time. This reduction in overall time and manual effort are clear advantages for ASP preparation. Additionally, the reproducibility of automation and the capability of programmable control to optimize protocols for different tissues and research needs are also benefits. Further, the ASP could also perform

SBEM immunostaining, as demonstrated for TEM [2]. Finally, since the enclosed ASP either vents into or can be contained in a fume hood, experimenter exposure to noxious reagents may be reduced.

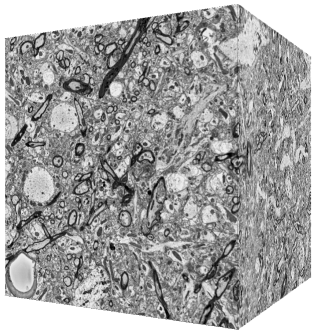
References:

[1] TE Strader et al., Microsc. Microanal **24** (2018), p. 1284.
[2] P Marques et al., Microsc. Microanal **24** (2018), p. 1300.



2

Reagent	Exchanges	Agitations	Time (min)
Glut/PFA	perfusion		5°C until process
Buffer Rinse	3	90	3
Tannic acid	1	450	15
$K_4[Fe(CN)_6]$	1	1800	60
H2O	5x3	450	15
TCH	1	1800	60
H2O	1	90	3
OsO4	1	900	35
H20	5x5	450	25
UrAc	1	1800	60
H2O	3x2	270	6
Lead Aspartate	1	900	30
H2O	3x5	135	15
25% EtOH	1	110	10.5
50% EtOH	1	110	10.5
75% EtOH	1	110	10.5
95% EtOH	1	150	10.5
100% EtOH	1	150	10.5
Acetone	2x10	540	10.5
25% Resin	1	300	10.5
50% Resin	1	300	10.5
100% Resin	3x10	360	31.5
Resin cure	1		Overnight
Total Automatic Processing Time			**7 hr 22 min**



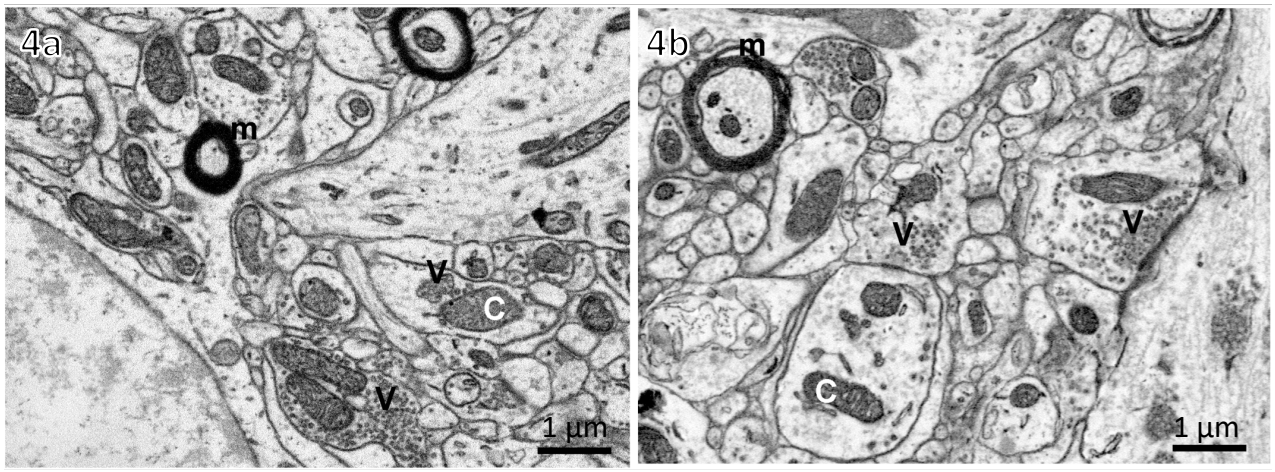


Figure 1. ASP-1000. Specimens in mPrep/s capsules (circled). Reagents in microwell plates (arrows).
Figure 2. Automated ASP protocol.
Figure 3. Cortex prepared with ASP. Perspective projection view from 350 70 um thick slices.
Figure 4. Manually prepared cortex (4a) and ASP prepared cortex (4b) from same brain: Myelin (m), synaptic vesicles (v) mitochondrial cristae (c).

Rapid Automated Preparation for Serial Block Face Scanning Electron Microscopy

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

1. Microscopy Innovations, LLC, Marshfield, WI, USA
2. University of Wisconsin, Departments of Pathobiological Science, Neurological Surgery, & Neuroscience, Madison, WI, USA
3. Renovo Neural Inc. and The Cleveland Clinic, Department of Neurosciences, Cleveland, OH USA

Introduction

Serial block face scanning electron microscopy (SBEM) creates exciting new possibilities for life science discovery. While SBEM imaging and analysis are extensively computer-controlled, SBEM sample preparation is intensely manual; using chemical fixation, heavy metal staining, and resin embedding methods developed for TEM, plus additional toxic and reactive steps. While manual TEM specimen preparation may be manually completed in 1-2 days, SBEM protocols generally require a week.

The mPrep ASP-1000 (ASP) provides rapid preparation of biological tissues for microscopy with repeated fluid exchanges that accelerate reagent diffusion and reaction in specimens. This enables the ASP to prepare mammalian tissues for TEM in just 1-3 hours [1]. Here we adapt a standard week-long manual SBEM protocol into an automated process that requires just 1 working day followed by an overnight resin cure.

Experimental

Brain cortex and liver were excised from perfusion-fixed 12-month old rats, then immersed and refrigerated in cacodylate-buffered glutaraldehyde-paraformaldehyde until subsequent preparation (Fig. 1). For ASP preparation ~1-3 mm tissue pieces were loaded into mPrep/s capsules by entrapping between the capsule and screen. Capsules were mounted on the ASP and reagents were dispensed into microwell trays sealed with pierceable covers (Fig. 1D-E).

The ASP executed the SBEM protocol (Table below) by aspirating successive reagents into capsules per programed operation. Mixing 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 with hands-on reagent changes over 3 days with 3-4 overnight steps.

Reagent	Reagent repeats	Mixing cycles	Time (min)
Karnovsky fix	Perfusion		Refrigerated
Buffer rinse	3	30/30/30	3
Tannic acid (some specimens)	1	450	15
OsO4 - KFeCN	1	1800	60
H2O	5	90/90/90/90/90	15
1% Thiocarbohydrazide	1	1800	60
H2O	5	90	3
OsO4	1	900	30
H2O	3	90/90/90/90/90	15
2% Uranyl Acetate	1	1800	60
H2O	3	90/90/90	9
Lead Aspartate	1	900	30
H2O	3	45/45/45	9
25% EtOH	1	110	9.17
50% EtOH	1	110	9.17
75% EtOH	1	110	9.17
95% EtOH	1	150	12.5
100% EtOH	3	150	12.5
Acetone	2	270/270	9
25% epoxy	1	300	10
50% epoxy	1	300	10
75% epoxy	1	300	10
100% epoxy	3	120/120/120&hold	30
	Total time (includes ASP motion & operations)		**~7.5 Hrs**

An FEI Teneo VolumeScope imaged blocks at 2.0 kV, 0.1 nA under high vacuum using the T1 detector for brain cortex, and 2.2 kV for liver.

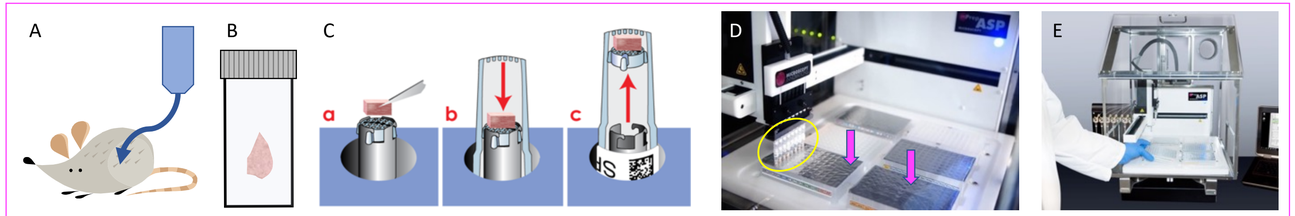


Figure 1: A) Rats were perfusion fixed. B) Organs pieces were excised & stored in fix at 5°C. C) Tissue oriented and entrapped in mPrep/s capsules using mPrep/s Workstation. C) Eight specimens in capsules (circled) ready for processing on ASP-1000. Reagents in sealed microwell plates (arrows). D) ASP-1000.

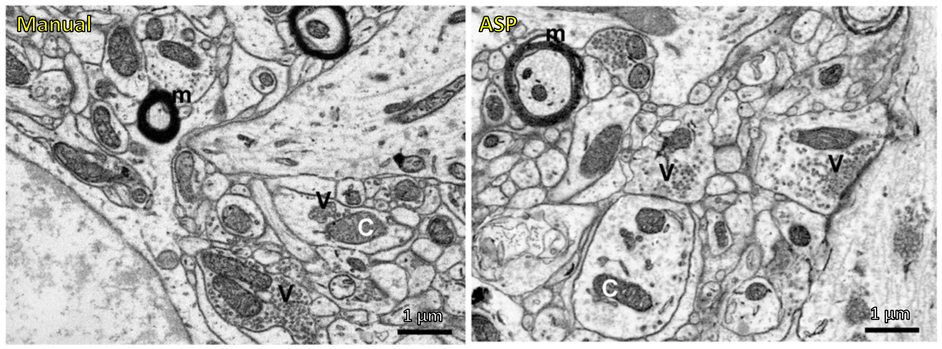


Figure 2: Manual & ASP prepared brain, SEM plane views. Myelin (m), synaptic vesicles (v), mitochondrial cristae (c).

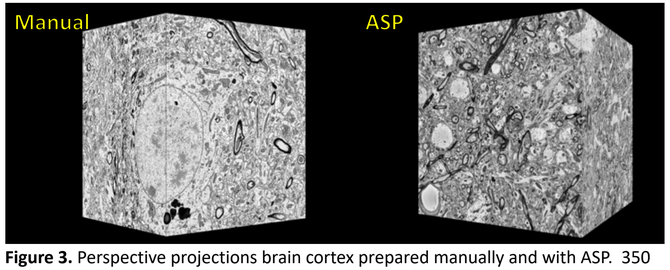


Figure 3. Perspective projections brain cortex prepared manually and with ASP. 350 70 nm thick slices, ~60 x 60 x 20 µm deep. Images acquired in 25 hr each.

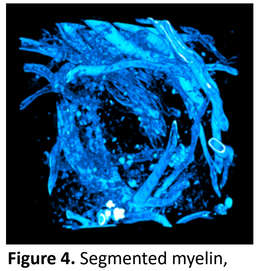


Figure 4. Segmented myelin, ASP prepared cortex.

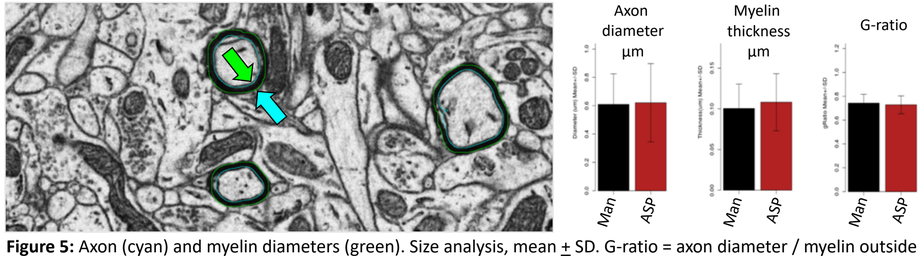
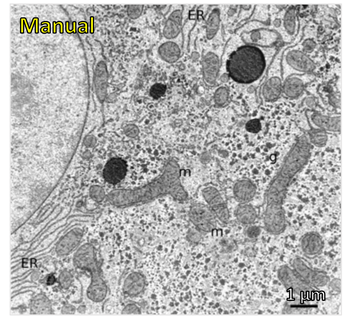


Figure 5: Axon (cyan) and myelin diameters (green). Size analysis, mean ± SD. G-ratio = axon diameter / myelin outside diameter. N= 4,894 axons measured.



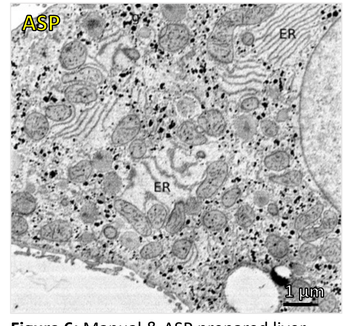


Figure 6: Manual & ASP prepared liver. Mitochondria (m), Endoplasmic reticulum (ER), Glycogen (g).

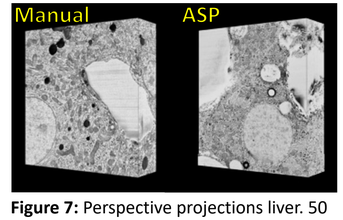


Figure 7: Perspective projections liver. 50 70 nm slices, ~20 x 25 x 3.5 µm deep.

Results and Discussion

The ASP-1000 prepared tissues in working day, followed by overnight resin curing. Operator effort required about 1 hour to load specimens and reagents prior to processing, and then to transfer resin infiltrated specimens to the oven for overnight polymerization. Manual vial processing required 13 hours of hands-on reagent exchanges over several days, and 3-4 overnight steps.

SBEM quality was compared for manual and ASP-prepared brain (Figs. 2-5) and liver (Figs. 6-7). Both provided high-quality specimens with good sectioning properties. Myelin (brain cortex) 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 comparable (Fig. 2). Volume rendering of segmented myelin provides 3D spatial information (Fig. 4). Axon and myelin dimensions were the same for manual and ASP prepared tissue (Fig. 5). Liver was similarly well-prepared with both manual and ASP processing (Fig. 6-7).

The ASP provided quality preparation in much less time with much less effort. Automated reproducibility and programmable control to optimize protocols are additional ASP benefits, including the potential to perform SBEM immuno labeling, as demonstrated for TEM [2]. Although only SBEM analysis is demonstrated here, the ASP may also be applied to Focused Ion Beam and array tomography SEM, since specimen preparation for these 3D EM modalities are similar.

Workflow

The ASP-1000 streamlines SBEM workflows in comparison to manual preparation methods:

- Specimens processed automatically in 1 day, instead of a week
- Operator time reduced to 1 hour
- Reduces handling and exposure to toxic reagents
- Eliminates specimen handling, including messy transfers in resin
- Provides automated consistency and reproducibility
- Protocols are readily modified to alter stains, perform en bloc immuno-gold labeling, or change other parameters [1-2].

With the ASP-1000, SBEM workflows are automated throughout:

Specimen preparation ⇒ SBEM imaging ⇒ Analysis

How is the ASP-1000 process speed so fast?

Chemical fixation, staining, and embedding are largely diffusion limited reactions. The ASP-1000 and mPrep/s capsules accelerate diffusion into specimens using gentle alternating fluid flow, with mixing cycles repeated as rapidly as every second.

References

[1] TE Strader, et al. Automated rapid preparation of tissue specimens for TEM pathology. Microsc. Microanal 24 (2018) p. 1284.

[2] P Marques, J Strong, *et* al, Optimization of Automated Immuno EM for Both Pre- and Post-Embedding Labeling. Microsc. Microanal 24 (2018) p. 1300.