Automated Specimen Preparation for Volume Electron Microscopy

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Volume electron microscopy (vEM) chemical specimen preparation typically requires 3-5 days to prepare while requiring 1-2 days of hands-on effort with toxic reagents. This slows progress and takes time away from imaging, interpretation, and analysis. The tedium and duration of manual prep can also cause inconsistencies that negatively affect reproducibility and automated AI image segmentation quality. We illustrate here how vEM labs use mPrep™ ASP™ Automated Specimen Processors (ASPs) to provide high-quality, consistent, and efficient specimen preparation: 1) The Knight Cancer Institute and Multiscale Microscopy Core at Oregon Health & Science University and, 2) The 3DEM Ultrastructural Imaging & Computation Core at the Lerner Research Institute of the Cleveland Clinic.

Specimens are prepared for vEM, TEM and other microscopies using ASPs and mPrep/s™ specimen capsules (Fig. 1). Specimens in mPrep/s capsules are attached to the 8-channel ASP head (Fig. 1A-B), with user-loaded reagents in microplates. Reagent timing and microfluidic agitation (Fig. 1D) is controlled by the ASP Dashboard using user-sharable and easily modified protocols (Fig. 1C). mPrep/s capsules adapt to different specimens and workflows (Fig. 1E-J). The 2 labs shown here typically prepare up to 4 specimens/capsule for a capacity of 32 specimens per preparation run (Fig. 1G). Others prepare up to 8 specimens/capsule, with 2 capsules per ASP channel (Fig. 1F) for a capacity of 128 tissue specimens [1].

Oregon Health & Science University (OHSU) specimens were prepared with 4.5 hour-long ASP protocols. Their prior studies compared 4.5-hour ASP to multi-day manual prep with brain and tumor specimens imaged with FIB-SEM, SBF-SEM and array tomography, reporting that ASP prep improved reproducibility, enabled deep learning image analysis, reduced operator effort, cut processing costs, and improved safety [2]. Here we show pancreatic and breast cancer FIB-SEM (Fig. 2) specimens as vEM stacks and as automated segmented images. ASP preparation provides consistent contrast that enabled automated segmentation that was trained from only 5% of manually annotated ground truth.

Cleveland Clinic specimens were ASP-processed in 8-hours. Prior studies compared 8-hour ASP-prep to 4-day manual prep of brain cortex, reporting that ASP results were equivalent or superior for staining, preservation, infiltration, sectioning, and imaging myelin, synapse, and mitochondria [1]. Here we show a novel toxic demyelination recovery assay in mouse corpus callosum. SBF-SEM vEM and AI-segmentation follows a single axon through its tortuous course as if in a single plane [3]. Segmentation then locates the nodes of Ranvier traced in single axons to calculate axon-myelin dimension G-ratios. High G-ratios in Fig. 3D might suggest remyelination, but vEM analysis indicates a pathologically swollen axon accumulating disrupted mitochondria and other organelles typical of impaired axonal transport.

In summary, we show here how two labs use ASPs for "hands-off" vEM specimen prep in just 4.5-8 hours (before resin curing), with results equivalent or superior to manual prep, while providing the staining reproducibility for consistent AI automated segmentation.

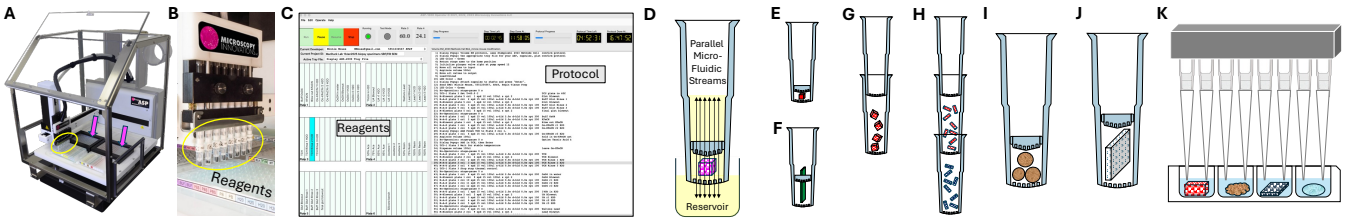

Fig. 1. mPrep ASPs (A-B) deliver reagents to specimens in mPrep/s capsules on the 8-channel ASP head (circled) from reagents in microplates (B). C) Dashboard controller. D) Bi-directional microfluidic reagent flow. E-J) mPrep/s capsules with ASP can process oriented specimens (E-F) or several specimens (G-I), culture substrates (J). ASPs also automate preparation of large specimens & coverslips (K) using pipetting.

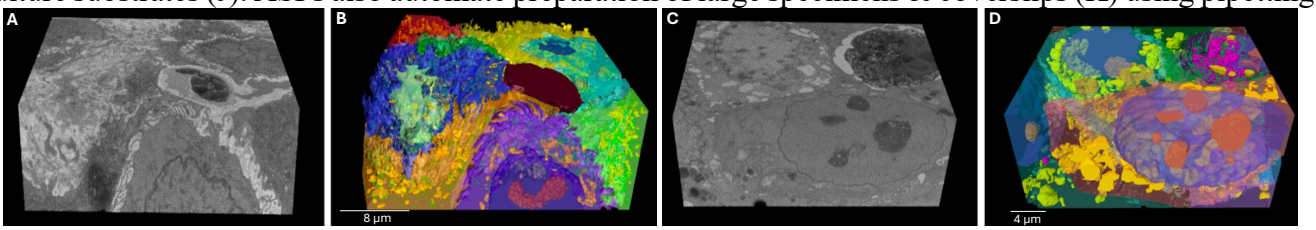

Fig. 2. Pancreatic and Breast Cancer FIB-SEM at OHSU. A-B) Human pancreatic ductal adenocarcinoma biopsy vEM stack (A) and automated segmented image (B). C-D) Human MCF7 breast cancer spheroid 3D culture (C) and segmented vEM stacks (D): Nuclei = dark blue. Nucleoli = dark orange, Mitochondria = yellow, Individual cell membranes various colors. FEI Helios G3 NanoLab DualBeam FIB-SEM.

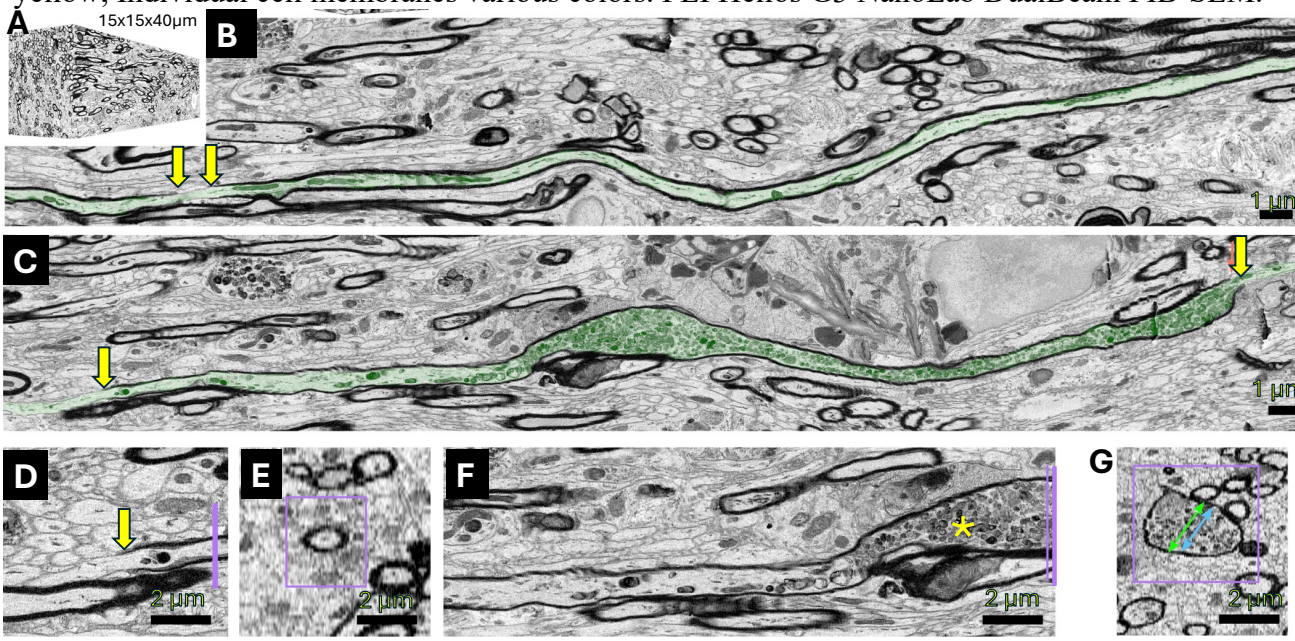

Fig. 3. Mouse Corpus Callosum Axon - Toxic Demyelination Assay at Cleveland Clinic. A) vEM stack. B) Intact axon (green) followed ~40 µm by digital reslicing. Arrows=Node of Ranvier paranodes. C) Axon (green) with isolated myelin internode (arrows=heminodes). D-G) Higher mag paranodal structures. D-E) Transverse axon resliced. Box in E=Plane of magenta line in D. F) Membranous debris (*). G) Transverse resliced magenta line shows distended ~2.5 µm diameter axon (arrow) compared with 1 µm in (D).

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3. Benson EK, *et al*. *Society Neuroscience* (2024) LBA20.

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Introduction

Volume electron microscopy (vEM) specimen preparation using chemical fixation, staining and embedding when done manually typically takes 3-5 days with 1-2 days of manual “hands-on” effort with toxic reagents. This slows progress and takes time away from imaging, interpretation, and analysis. Further, the complexity and tedium of manual preparation can lead to inconsistencies that negatively affect result reproducibility including with automated image segmentation.

Methods

We illustrate here how mPrep™ ASP™ Automated Specimen Processors (ASPs) are used in two vEM labs:

1. The Knight Cancer Institute and Multiscale Microscopy Core, Oregon Health & Science University. Specializing in cancer and biomedical research. Uses an mPrep ASP-2000 for specimen preparation.
2. The 3DEM Ultrastructural Imaging & Computation Core, Lerner Research Institute, Cleveland Clinic. Specializing in pre-clinical neurological imaging using vEM and computational analysis. Uses an mPrep ASP-1000 for specimen preparation.

ASPs and mPrep/s specimen capsules (Fig. 1) enable preparation of different specimen types for imaging applications including vEM, TEM, SEM, XRM, & CLEM. Specimens in mPrep/s™ capsules are attached to the 8-channel ASP head (Fig. 1A-B), with user-loaded reagents placed in 96-well or 12-well microplates on the ASP deck. Reagent timing and microfluidic agitation (Fig. 1D) is controlled by user-shared, modified, and easily-created protocols run on the ASP Dashboard (Fig. 1C).

mPrep/s capsules are used in different ways for different specimen types and workflows (Fig. 1E-J) . Specimens can be orientated before embedding (Fig. 1E-F) or processed without orientation (Fig. 1G-I), with a single preparation run process capacity of up to 64 vEM and 128 TEM tissue specimens [1-2]. Cell culture substrates up to ~4.2 x 9 mm can be processed in mPrep/s capsules. Specimens larger than 4+ mm can be ASP-processed in 24-well plates using pipette tip reagent delivery and agitation [3] (Fig. 1K).

Results

Oregon Health & Science University

Left column examples include:

- Human biopsy pancreatic ductal adenocarcinoma - FIB-SEM with automated segmentation
- Human MCF7 breast cancer 3D spheroid cultures - FIB-SEM with automated segmentation
- Marmoset Brain - SBF-SEM & FIB-SEM
- Mouse Pancreas - SBF-SEM & FIB-SEM.

These specimens all were processed with similar ASP vEM protocols in less than 5 hours (Table). Prior studies at the Multiscale Microscopy Core (MMC) compared 5-6-hour ASP to multi-day manual preparations with brain and tumor specimens, imaged with FIB-SEM, SBF-SEM and array tomography. These studies reported that ASP preparation improved reproducibility, provided images suitable for deep learning image analysis, greatly reduced operator effort, cut processing costs, and improved safety by cutting toxic reagent handling [1,4].

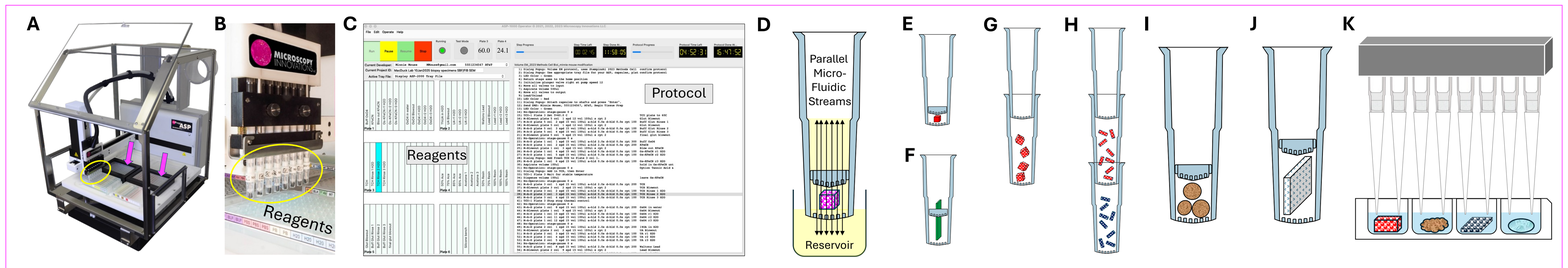
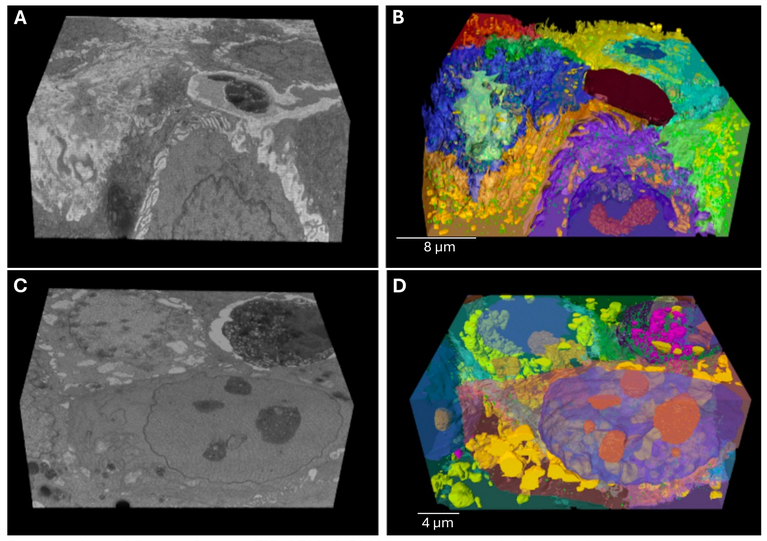
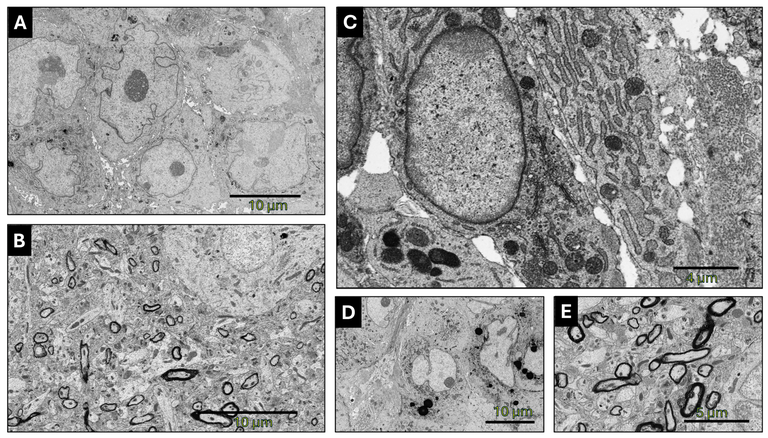


Figure 1: ASP™-1000 & ASP™-2000 Automated Specimen Processors. **A-B**) ASPs deliver reagents to specimens in mPrep/s capsules on the 8-channel ASP head (circled) from reagents in microplates (**B**). Arrows show two 0-100°C controlled reagent plates on ASP-2000. **C**) Intuitive user-modifiable & sharable protocols. **D)** Bi-directional microfluidic flow in mPrep/s capsules provides rapid & uniform reagent infiltration. **E-F**) mPrep/s specimen capsules can orient individual specimens using mPrep/s screens, or **G-H**) entrap several specimens without screens, up to 8 specimens/capsule. Other specimen loading options include; **I**) Multiple specimens, e.g. spheroids, **J**) Cell culture substrates/coverslips up to 4.3 mm wide. **K**) Specimens >4 x 9 mm, e.g. large tissue pieces & whole coverslips prepared in 24-well plates using pipetting [3].

Oregon Health and Science University



Pancreatic and Breast Cancer FIB-SEM. A-B) Human pancreatic ductal adenocarcinoma biopsy vEM stack (**A**) and automated segmented image (**B**). **C-D**) Human MCF7 breast cancer spheroid 3D culture (**C**) and segmented vEM stacks (**D**). **B & D**) Segmentation trained from only 5% of manually annotated ground truth: Nuclei = dark blue. Nucleoli = dark orange, Mitochondria = yellow, Individual cell membranes various colors. ASP-2000 prep with modified Hua protocol <5 hrs. FEI Helios G3 NanoLab DualBeam FIB-SEM. OHSU Brenden-Colson Center for Pancreatic Care, Center for Spatial Systems Biology, Multiscale Microscopy Core & Knight Cancer Institute.

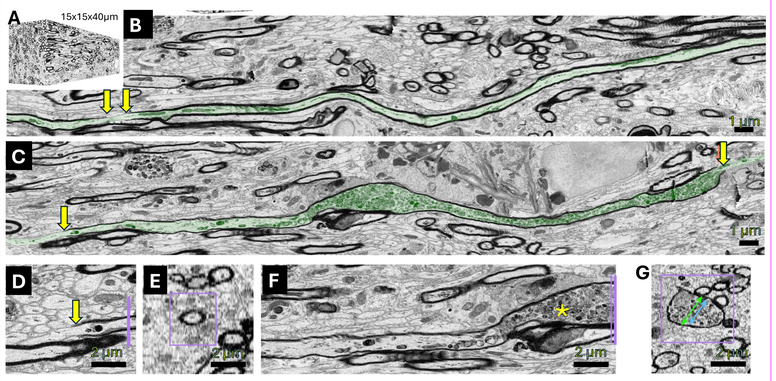


Brain and Pancreas. vEM processed in <5hrs**.** SBF-SEM (**A & B**), FIB-SEM (**C-E**). **A**) Mouse Pancreas. **B**) Marmoset Brain. **C-D**) Mouse Pancreas. **E**) Marmoset Brain. MMC, OHSU.

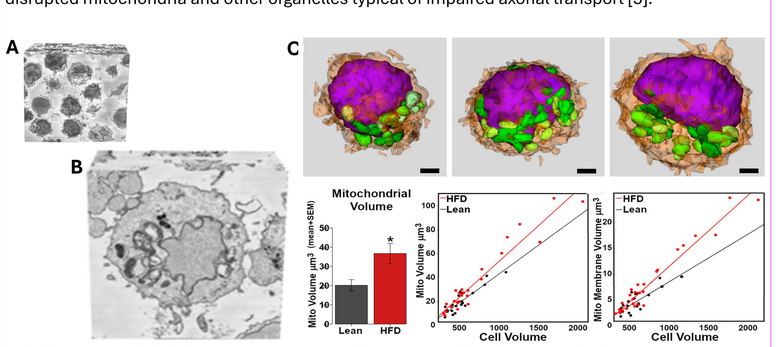
vEM Protocol	Timing	
Reagents	**Manual**	**ASP-2000**
Rinse - sodium cacodylate buffer	15min	9min
2% OsO_4 in buffer	90min	13min
2.5% $K_3[Fe(CN)_6]$ in buffer	90min	13min
Rinse - water	25min	9min
1% thiocarbohydrazide (TCH) 60C	45min	13min
Rinse - water	25min	9min
2% OsO_4 in water	90min	13min
Rinse in water	25min	9min
1% UA aqueous (manual = overnight)	~720min	13min
Rinse - water	25min	9min
Walton's lead	120min	13min
Rinse - water	25min	9min
Acetone dehydration Manual: all steps x2: 50%, 75%, 85%, 95%, 100% ASP: 50%, 75%, 85%, 95%, 100% x 3	50min	21min
Resin infiltration (manual = overnight) Bench: Acetone:Resin 1:1, 1:3, Pure resin x5 ASP: Acetone:Resin 1:1, 1:3, Pure resin x3	~720min	123min
Total	**2 days 3 hrs 20 min**	**4 hr 36 min**

vEM Preparation Protocol for ASP vs. Manual bench: Multiscale Microscopy core (MMC), Oregon Health and Sciences University. (Cleveland Clinic uses similar 7-8 hr vEM ASP protocols.)

Cleveland Clinic



Mouse Corpus Callosum Axon & Myelin Toxic Demyelination Recovery Assay - SBF-SEM. A) vEM stack, ASP-1000 prepared in ~7hrs. **B**) Intact axon (green) followed ~40 µm by digital reslicing. Arrows: Node of Ranvier paranodes. **C**) Axon (green) with isolated myelin internode (arrows=heminodes). **D-G**) Paranodal structures at higher magnification. **D-E**) Axon resliced transversely to measure axon diameter & thickness. Box in E = Plane of magenta line in D. **F**) Axon containing membranous debris (yellow asterisk). **G**) Reslicing transversely at magenta line shows distended ~2.5 µm diameter axon (cyan arrow) compared with 1 µm in (**D**). Ratio of external myelin diameter (green arrow) to axon diameter (G-ratio) is 0.85, compared with 0.71 in (C). High G-ratio might suggest remyelination, but vEM analysis indicates pathologically swollen axon accumulating disrupted mitochondria and other organelles typical of impaired axonal transport [5].



B-Cell Pellets – SBF-SEM: Splenic mouse B-Cells were pelleted, enrobed in low melting agarose, and ASP-1000 processed for ~7.5hrs. **A**) SBF-SEM pellet volume image. **B**) Individual B-cell, **C**) Segmentation and analysis of individual B-Cells [2,6]. Mitochondria = green, Nucleus = Purple.

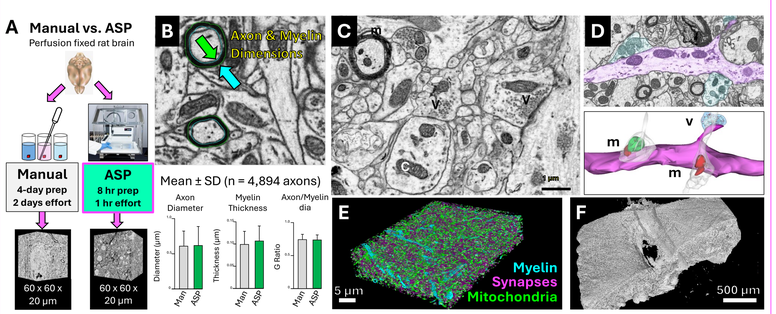


Figure 4: Rat Brain SBF-SEM: A) Perfusion-fixed rat brain cortex was prepared manually and by ASP-1000. Both provided high-quality staining, preservation, infiltration, sectioning, and imaging of myelin, synaptic vesicles, and mitochondrial cristae. **B**) Axon and myelin dimensions were statistically equivalent. **C**) Single plane from volume image, mitochondria (m), cristae (c), synaptic vesicles (v). **D**) Dendrite (magenta) single plane image shows synapses (red & green), and in 3D volume image. **E**) AI segmentation, identification & quantification. **F**) Correlative XRM of block from same brain [2,7].

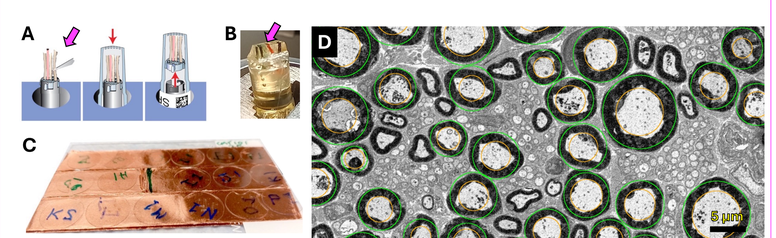


Figure 5: Peripheral Nerves: A) Three nerves oriented in one mPrep/s capsule with fiduciary thread (arrow), then ASP-prepared using a TEM-like ASP protocol with 90 minutes OsO_4 and then **B**) resin embedded in mPrep/s capsule. **C**) 1 µm thick sections mounted, UA & Pb stained on coverslips, then arrayed on copper tape. **D**) SEM of auto-segmented axons for G-ratio measurement [2,7].

Results

Cleveland Clinic 3DEM Core

Right column examples include:

- Mouse brain corpus callosum, auto-segmented nodes of Ranvier traced in single axons - SBF-SEM [5]
- Splenic mouse B-Cell pellets, auto-segmented - SBF-SEM [6]
- Rat brain cortex SBF-SEM: 4-day manual vs. 8-hour ASP prep. Auto-segmented & G-ratio analysis – SBF-SEM [2,7]
- Rat brain cortex SBF-SEM auto-segmented [2,7]
- Rat brain cortex correlative XRM
- Peripheral nerve auto-segmented & high throughput G-ratio analysis -SEM [2,7].

Discussion

We have shown how two labs use mPrep™ ASP™-1000 and ASP™-2000 Automated Specimen Processors for vEM preparation. Both labs report "hands-off" vEM specimen prep in 5 to 8 hours (before resin curing), with results equivalent or superior to manual prep, with staining reproducibility for consistent AI automated segmentation, and the safety of reduced reagent handling. Other reports have shown ASP preparation of a wide range of specimens for vEM & TEM that include planaria [8-9], many, many more vertebrate tissues, biopsies and cells [e.g. 2,3,6,10,11], and pre-embedding and on-grid immunolabeling [11-12].

ASP™ Automated Processors

- Nearly any EM specimen prep task
- TEM, vEM, IGL, SEM, XRM, Freeze-substitution steps >0°C (ASP-2000)
- Share, modify & create protocols
- Prepare 1-128 specimens at a time
- As little as 40µl reagent/specimen/step
- 5-10x faster prep than manual
- 5-10-minute set-up and clean-up
- Purpose-built for reactive EM reagents: OsO_4, RuO_4, TCH, solvents, resins, unlike aqueous reagent-only biology lab robots
- A decade of proven durability.

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