Innovations & Applications of Manual & Automated Specimen Preparation for Volume Electron Microscopy

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Introduction

Specimen preparation for volume electron microscopy (vEM) is demanding. Manual preparation typically requires 3-5 days with one or more full days of "hands-on" reagent processing. This slows research progress and ties-up staff Plus, the tedium and complexity of manual prep can reduce consistency which can affect reproducibility.

mPrep System

The mPrep System was created to reduce preparation time and effort, cut reagent consumption, improve reproducibility, and be highly adaptable. mPrep™ specimen and grid capsules, for use with hand-held pipettors, were introduced in 2011. The ASP™-1000 Automated Specimen Processor was introduced in 2015, followed by the ASP-2000. Figure 1 illustrates typical ASP™ and mPrep/s™ capsule workflows for vEM specimen prep. Figure 2 illustrates multiple ways that mPrep/s capsules can be used for different applications. Figure 3 shows how ASPs enable efficient and rapid specimen prep with repeated directed reagent flow to infiltrate reagents into specimens, and with near-zero carryover.

Automated vEM Preparation

The first report using an ASP for vEM specimen prep was by McClain et al. [1] who automated an "Ellisman" protocol [2] to prepare planarian flatworms in just 1.5 days, including resin curing, in comparison to 7-day manual preparation for these difficult-to-infiltrate specimens. This report described equivalent quality with single-section SEM imaging. McClain et al. followed this with a serial block face SEM (SBF-SEM) report demonstrating comparable quality with ASP and manually-prepared blocks [3]. Goodman et al. and Benson et al. [4-5], also compared manual to ASP prep for SBF-SEM with brain and liver, reporting at least comparable prep quality with the ASP, achieved in about 1/5 the time with 1/10 the hands-on effort: vEM prep in just 8 automated hours and 1-hour effort, compared to 4 days manual prep with 2-days hands-on effort. Stempinski et al. [6] recently reported using an ASP-2000 to simultaneously prepare up to 4 brain and tumor specimens/capsule for vEM, enabling up to 32 tissue pieces to be prepared in 7 hours (with 2.5 hr technician time) compared to 2.5 days (with 5.5 hr technician time) for their manual protocol, prior to resin curing.

Brain vEM with ASP prep

Figure 4 compares rat brain prepared manually and with an ASP-1000. Preparation is comparable for staining, infiltration, and SBF-SEM sectioning. Quantitative imaging of axon size and myelin thickness are identical, with ASP-prepared specimens enabling AI segmentation [4-5]. ASP blocks from this preparation can also be imaged with X-ray microscopy for correlative imaging of larger 3D volumes (Figure 4F).

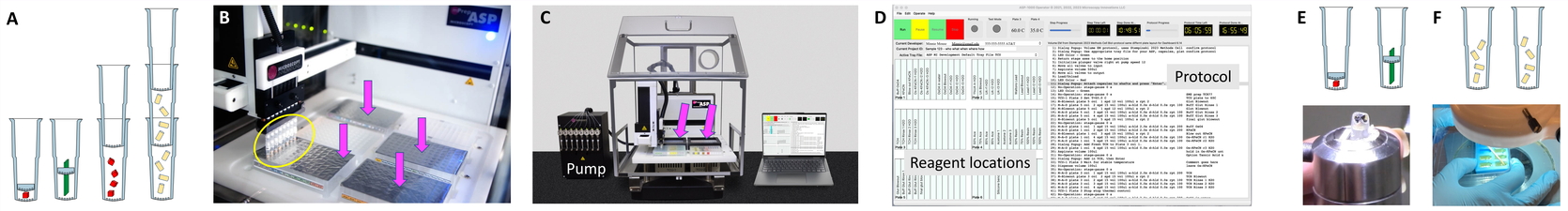

Figure 1: mPrep™ System Workflows: ASP™-1000s and ASP-2000s reduce personnel effort and can perform nearly any vEM or TEM protocol: A) Specimens are loaded into mPrep/s capsules. Can orient in individual capsules, or load 1-8 specimens per capsule for high capacity. B) Capsules are attached to 8-channel ASP™-1000 head (circled). Capsules can be stacked on each pipettor shaft to multiply capacity, enabling simultaneous preparation of up to 32 tissue specimens for vEM [6] and up to 128 for TEM [7]. Preparation reagents are shown in 4 sealed microwell plates (arrows) & 2 open microplates on 6-plate deck. C) ASP™-2000 with fume enclosure, pump module & laptop controller. ASP-2000 has two 0-100C temperature-controlled microplates (arrows). D) ASP Dashboard enables virtually any protocol. Top control bar shows status, timing, and temperature (ASP-2000 only). Reagent locations are shown for the 6 microplate positions. E) ASP-processed specimens can be embedded and sectioned in mPrep/s capsules to provide specimen orientation and reduce handling, or F) specimens can be removed and embedded in conventional molds.

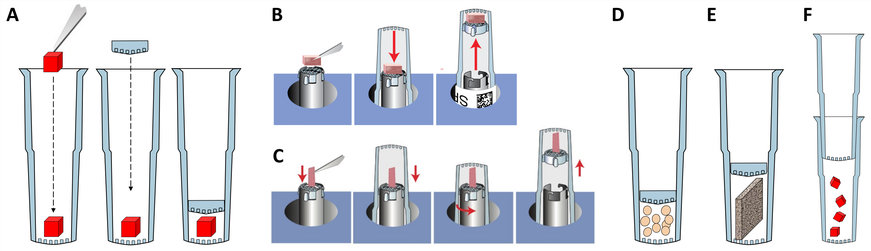

Figure 2: Specimens in mPrep/s capsules: Capsules are used differently for different applications and workflows. A) Tissue specimens, pellets, etc. are placed in capsules and entrapped with mPrep/s screens using an insertion tool (not shown). B) Use mPrep Workstation to orient specimens by entrapping between capsule bottom and screen, or C) orient long specimens (e.g. nerve fibers) by clamping the back of the specimen with screen. D) Entrap several specimens. E) Planar specimens, e.g. cell culture substrates, can be oriented across ~4.3 mm diameter. F) A top capsule can cap specimens in bottom capsule without a screen. Use for many applications including cryo, since screens can't be inserted at cryo temps. Capsule pores are 300 µm. Enrobe small specimens (e.g. agar) to entrap in capsules.

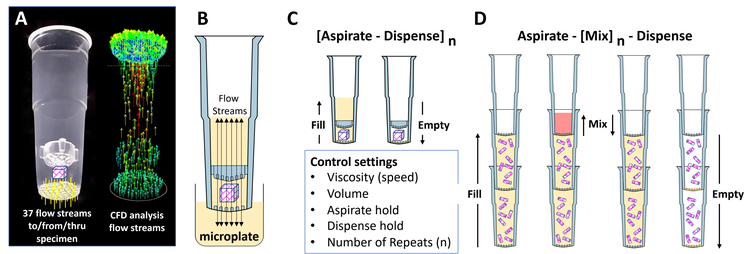

Figure 3: Rapid Reagent Infiltration: A) Bidirectional reagent flow is directed to and thru specimens from 37 parallel flow streams to accelerate infiltration. B) Reagent is delivered from 12 or 96-well microplates. Agitation is tailored for application:
C) ***Aspirate – Dispense*** completely fills and then empties capsules. Can repeat every half second for 100's of times (control settings). D) ***Aspirate-Mix-Dispense*** fills to immerse specimens, then aspirates and dispenses additional reagent for gentle bidirectional mixing to drive reagent to/thru specimens for up to 100s of repeats (control settings), then dispenses. Carryover volume after dispense approaches zero.

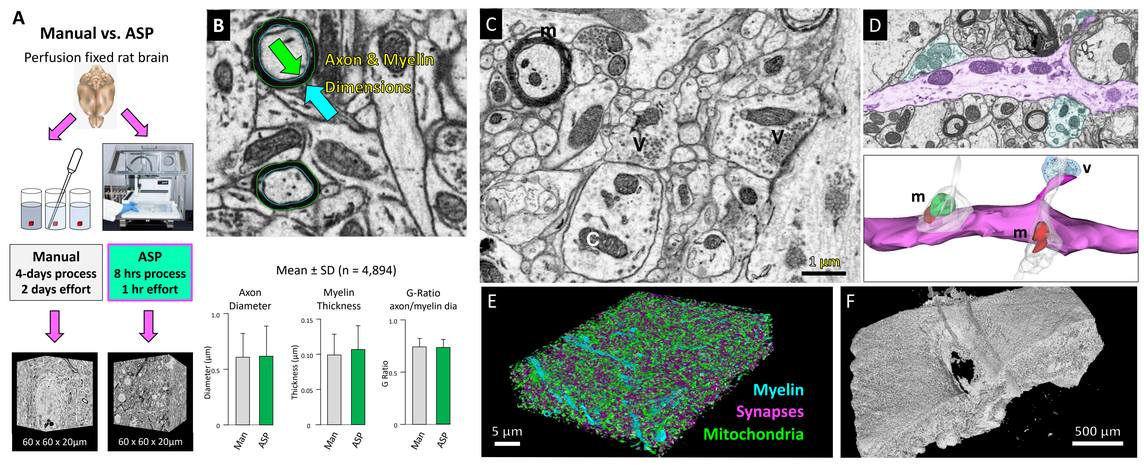

Figure 4: Brain vEM [5-6]: A) The same GA-PA perfusion-fixed rat brain was prepared manually and ASP-1000. Both provided high-quality staining, preservation, infiltration, sectioning, and imaging of myelin, synaptic vesicles, and mitochondrial cristae. B) Axon and myelin dimensions were statistically 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 identification & quantification enabled with robotically uniform preparation. F) Correlative X-ray microscopy provides image of much larger volume.

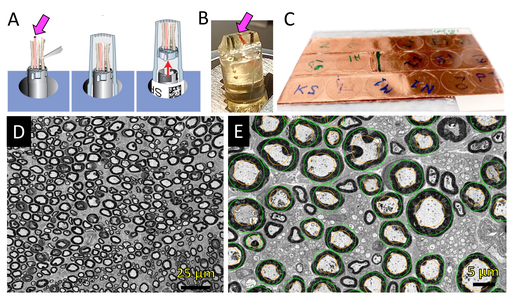

Figure 5: Peripheral Nerve: A) Three nerves oriented in mPrep/s capsule with fiduciary thread (arrow), then ASP-prepared for vEM (Figure 1) and B) resin embedded in the capsule (arrow). C) 1 µm sections are mounted and UA-Pb stained on coverslips, then arrayed on copper tape. D-E) SEM images with auto-segmented axons (E).

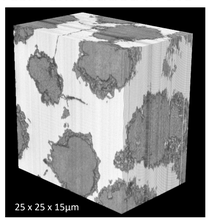

Figure 6: Lymphocyte vEM: Lymphocytes were pelleted, enrobed in low melting agarose, and then ASP processed for vEM.

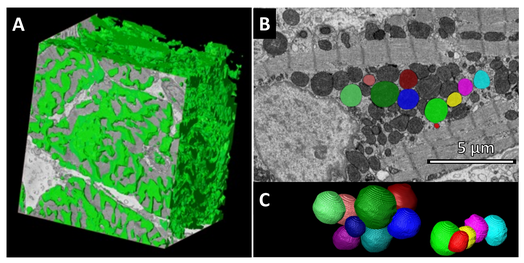

Figure 7: Heart right ventricle vEM, from neonate ICU hyperoxia model [8]: A) SBF-SEM 25 µm^3 cube, segmented to show volume fraction (52.7%) of mitochondria (green)in right ventricle. B) Single slice with segmented colored mitochondria. C) 3D rendering of segmented mitochondria in B. Specimens were ASP prepared from perfusion-fixed rats with a 7.5 hr protocol before resin curing. SBF-SEM at Thermo Fisher Nanoport, Hillsboro, OR.

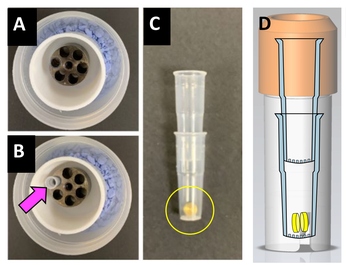

Figure 8: HPF & FS Workflow [9]: A) Teflon cup with desiccant holds mPrep CPD holder to B) receive capsule bottom (arrow) to entrap HPF planchette. C) Capsules entrapping planchet (circled). D) Diagram shows capsules to entrap planchets also fit into cryovial.

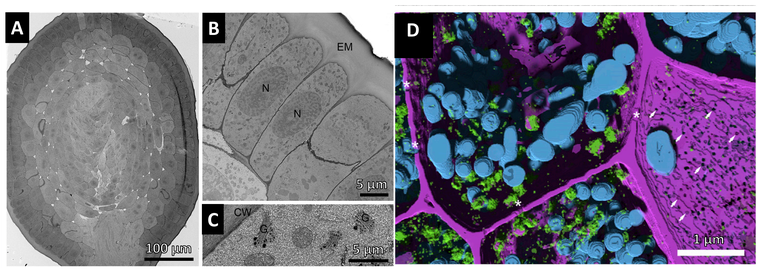

Figure 9: SBF-SEM HPF H. vulgare root FS aqueous OTO protocol, excerpted from [9]: A) Root cross-section near apical meristem. B) High-resolution slice from (A), note outermost protoderm cells with nuclei (N), electron-dense cell wall with thick extracellular matrix (EM) on root exterior. C) Protoderm cell with well-stained Golgi, cell wall, and trans-Golgi vesicle contents. D) 3D rendering using Sensor 3D of rapidly dividing cells in ground meristem with well-contrasted cell walls, mitochondria (cyan), Golgi and secretory vesicles (green), cell wall (magenta), and intersecting plasmodesmata voids (asterisks).

Peripheral Nerves

Therapeutic trials typically require imaging 24-62 nerves. Cleveland Clinic expedites sample prep by orienting 3 nerve specimens in each mPrep/s capsule, with a red thread fiduciary. ASP specimen prep uses ~4-hr TEM-like protocol with 90 minutes OsO_4 (Figure 5). One µm sections are mounted on coverslips, stained with methanolic uranyl acetate and lead citrate, and arrayed on copper tape for automated SEM imaging in 24-48 hrs. Axon measurements are auto-segmented for therapy evaluations.

Lymphocyte vEM

Lymphocytes at the Cleveland Clinic were pelleted, enrobed in low melting agarose, and ASP processed for vEM in ~8 hrs for vEM imaging (Figure 6).

Heart vEM

A respiratory neonate hypoxia study of heart right ventricle mitochondria (Figure 7) was performed at the University of Wisconsin (Dept. Medicine) with a 7.5 hr ASP vEM protocol. Imaging used a ThermoFisher VolumeScope (low vacuum 2.5 kV, 0.1 nA backscatter) and Amira Software image processing, both done by ThermoFisher Scientific [8].

High Pressure Freezing vEM

Figures 8-9, excerpted from Belanger et al. [9] illustrate high-pressure freezing (HPF) and freeze substitution (FS) using mPrep/s capsules without screens. This provided a facile means to entrap HPF planchets and specimens at cryo temperatures, and that prevents plant specimens from floating, which can lead to poor processing. mPrep/s capsules are held in high thermal capacity mPrep CPD holders during freeze substitution. This report used manual vEM prep. With an ASP-2000, portions of the vEM protocol above 0 C could be automated.

Summary

ASPs and mPrep/s capsules are adaptable to a wide range of vEM applications to improve throughput, reduce effort, and enhance reproducibility in order to advance vEM structural biology.

References

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