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Meeting-report

Automated Specimen Preparation for Electron Microscopy

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Biological electron microscopy of specimens prepared with chemical fixation, heavy metals, and resin embedding has been around for over 50 years and continues to revolutionize biomedical and botanical structural biology. This includes research and clinical diagnostic TEM and is especially true with the recent explosive growth of volume EM, which uniquely provides molecular-resolution structural biology in near-millimeter volumes while enabling correlative light and x-ray microscopy [1-4].

While TEM and vEM microscopes are highly automated, chemical specimen prep in most EM labs requires lab staff to sequentially immerse biospecimens in fixatives, labels, heavy metals, solvents, and embedding resins. Manual TEM prep typically takes 1-3 days while vEM takes 4-5 days. Manual prep can lead to errors and may not provide the consistency required for clinical diagnoses and for the artificial intelligence image segmentation and quantification that is becoming essential for understanding the 100 um^3 and larger datasets obtained from vEM for neuro-connectome, cancer, and other disciplines [1-4].

Microscopy Innovations LLC introduced the mPrep™ ASP™-1000 Automated Specimen Processor in 2015 and the ASP™-2000 in 2023 with next-generation ASP control software to meet the needs of most all EM labs [5-6]. ASPs (Fig. 1) have a base unit where specimens entrapped in mPrep/s capsules (or grids in mPrep/g capsules, not shown) are attached to the pipetting head. The ASP moves the pipetting head to microplates containing user-loaded reagents, where the precision pump aspirates reagents into the capsules to immerse the specimens (or grids). Specimens are loaded into mPrep/s capsules in multiple ways for different needs (Fig. 1C). The software-hardware engineering and specimen and grid capsules enable ASPs to meet the wide breadth of specimen and TEM grid preparation for most EM labs [2-7].

mPrep/s capsules use computational fluid dynamic design to provide dozens of parallel flow streams that gently drive bi-directional reagent infiltration through specimens. Combined with 10s to 100s of repeated aspirate-dispense cycles at up to ~1 cycle/second, a 2 mm thick specimen can be fully OsO_4 postfixed-stained in minutes [5-6]. This rapid infiltration alone leads to unprecedented specimen preparation times (Fig. 2), with preparation speed further accelerated by near-zero carryover between reagents. Many tissues have been prepared for TEM in just 1 hour from aldehyde rinse to resin curing [5-6] when using single capsules with screens (Fig.1C). Rapid prep is similarly achieved for vEM, reducing times from days to just 6-8 hours [2-6].

Large numbers of specimens are prepared by placing multiple specimens in each capsule, and even stacking two layers of specimen-containing capsules when required. The ASP protocol processes these by first fully immersing them, and then agitating them with directed bi-directional mixing while keeping them fully immersed, followed by full dispensing before moving to the next reagent. This reagent mixing function leverages the high-volume capacity and precision of the ASP pump. This method prepares up to 128 clinical biopsy segments in only 3.5 hours [5-6], and up to 64 vEM specimens in 6-8 hrs [2-3]. Reagent consumption is only ~5 ml at each protocol step for all specimens, thus only 40 µl/specimen when processing 128 specimens. This minimizes reagent purchase costs, waste, and user exposure. Comparable minimal reagent consumption has also been reported for immuno-gold labeling of TEM specimens or TEM grids, consuming only ~40 µl per specimen or 2 grids of these expensive reagents [7].

The user-friendly ASP Dashboard with its underlying hardware and software enables users to create preparative protocol by specifying the reagent sequence with natural language protocols that specify, 1) What reagent, 2) How long, 3) What agitation, and 4) What temperature (ASP-2000 model only). Many ASP labs modify or use already existing protocols since protocols are easily user-shareable, with dozens already created for most tissues, cell pellets, model organisms, and more, for applications that include TEM, vEM, immuno-gold labeling, and others [2-7]. Note that such protocol sharing aids research reproducibility. ASP setup requires just minutes thus minimizing hands-on time (Fig. 2), and then the ASP operates without user intervention allowing staff to focus on other tasks. However, some users program in Alerts (dialog popup, sound, signal light, SMS) if they wish to add labile or volatile reagents mid-protocol. In summary, ASPs efficiently and rapidly automate nearly any TEM and vEM protocol, freeing EM personnel from laborious manual preparation while improving consistency and reliability as needed for clinical and research TEM, vEM, AI image analysis, and other applications.

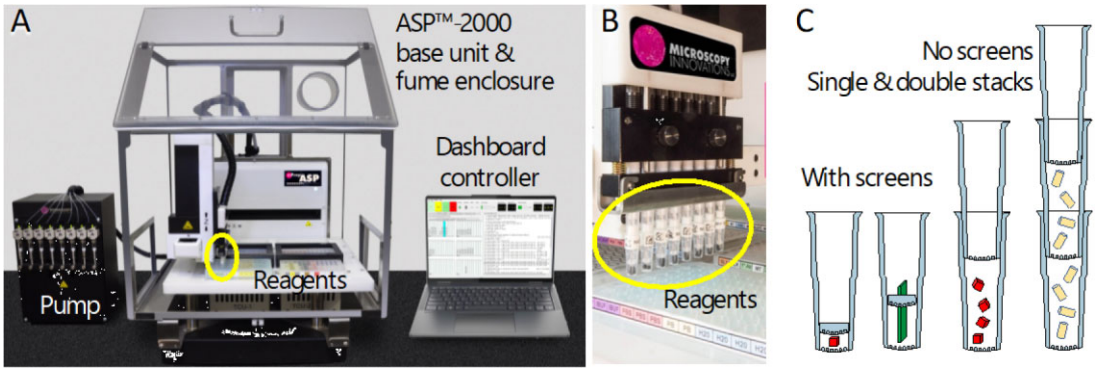

Fig. 1. A) ASP-2000 automated specimen processor with pump, fume enclosure, and Dashboard controller. mPrep/s specimen capsules attach to an 8-channel pipetting head (circled) which aspirate reagents from microplates. B) mPrep/s capsules on ASP head (circled) & reagent-containing microplates. C) Capsules with mPrep/s screens can entrap and optionally orient single specimens for embedding. Stacking capsules without screens can process multiple specimens. Double stacking capsules increase ASP process capacity. For TEM and vEM, specimens will usually be flat-embedded.

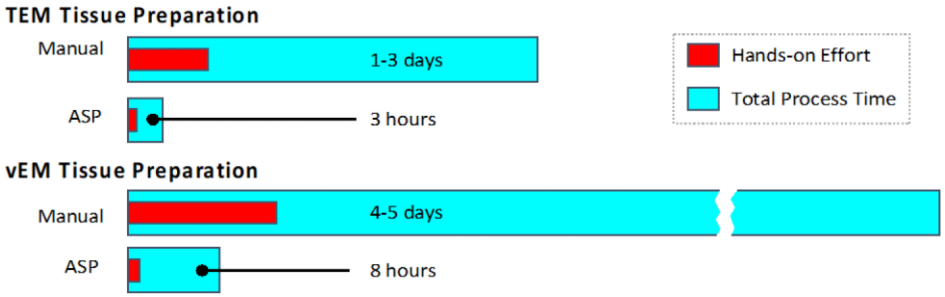

Fig 2. ASP and manual specimen preparation efficiency. Typical preparation times are shown for manual and ASP preparation for TEM and vEM.

References

1. LM Collinson *et al.*, *Nature Methods* **20** (2023). doi.org/https://doi.org/10.1038/s41592-023-01861-8
2. ES Stempinski *et al.*, *Methods Cell Biology* **177**:1 (2023). doi.org/https://doi.org/10.1016/bs.mcb.2023.01.009
3. GJ Kidd and EK Benson, *Microsc Microanal* **29** (2023), p. 1083. doi.org/https://doi.org/10.1093/micmic/ozad067.557
4. JL Riesterer *et al.*, *Microsc Microanal* **29** (2023). doi.org/https://doi.org/10.1093/micmic/ozad067.554
5. SL Goodman *et al.*, *Microscopy Today* **32**:1 (2024), p. 16. doi.org/https://doi.org/10.1093/mictod/qaad108
6. SL Goodman and JW Percival, *Microsc Microanal* **29** (2023), p. 2062.
7. P Marques *et al.*, *Microsc Microanal* **24** (2018), p. 1300. doi.org/https://doi.org/10.1017/S1431927618006980

Automated Specimen Preparation for Electron Microscopy

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Introduction

Biological electron microscopy (EM) of specimens prepared with chemical fixation, heavy metals, and resin embedding has advanced bioscience for 50 years and still continues to revolutionize structural biology and many other areas. This includes research & clinical TEM, the rapidly growing field of volume EM (vEM) [1-4], scanning EM, freeze substitution cryo-EM, and correlative light & x-ray microscopies.

Problem

EM imaging and analysis workflows are becoming increasingly automated, yet EM specimen prep has remained intensely manual in most labs. Chemical prep for TEM typically takes days of manual effort, and vEM requires nearly a week. This is a problem in today's EM labs, due to:

1) The growing shortage of well-trained bioEM scientists (81% MS or PhDs) as many retire (44% now aged 51 years or older), with few replacements available (47% of core lab staff report never training a novice), and frequent reports of difficulty attracting new staff [5-6].
2) Specimen prep ties up staff and displaces other work: Imaging, analysis, client/collaborator support, publishing, project & personnel management, microtomy, etc.
3) ***EM staff have too much work and too little time [5-7].***

Solution – mPrep Automated Specimen Processors

Purpose-built mPrep™ ASP™-1000 & ASP™-2000 Automated Specimen Processors (Figure 1), and mPrep specimen and grid capsules (Figure 2) meet the demanding workflow needs of small and large EM labs [8]:

- Fully automated walk-away prep for TEM, vEM, immuno-labeling, SEM, & freeze-substitution steps above 0°C.
- Any protocol: Up to 72 reagent steps without user action.
- Preparation speeds are typically 5-10x faster than manual.
- Prepare just 1 or up to 128 (tissue) specimens at a time.
- Low reagent consumption: As little as 40µl/specimen/step.
- Use your own protocols or protocols shared by other labs.
- Encapsulated specimens & grids cut loss or damage risk.
- Easy efficient 5-10-minute set-up and clean-up.
- Safer: Minimizes toxic reagent handling.
- Automated consistency and reproducibility.
- Automated documentation to ease admin tasks.
- Durability: ASPs are purpose-built for reactive EM reagents: OsO_4, RuO_4, thiocarbohydrazide, solvents, resins, activators, and other reactive reagents - unlike lab robots built for aqueous molecular biology reagents. ASP durability has been proven over nearly a decade.

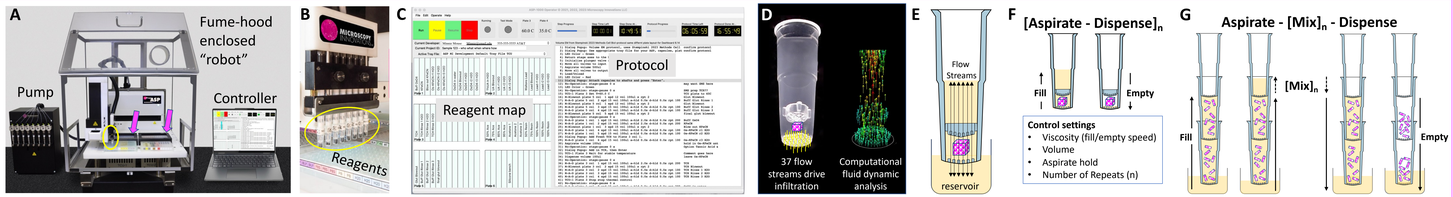

Figure 1: Purpose-built Automation. ASP™-1000 and ASP™-2000 Automated Specimen Processors are purpose-built for EM. **A)** ASP "robot" and pump for all EM reagents. **A-B)** Specimens (or grids) in mPrep capsules are loaded onto 8-channel head (circled). 12- or 96-well microplates hold reagents on 6-plate deck. Two microplates provide 0-100°C thermal control with ASP-2000 (arrows). **C)** Dashboard provides protocol control, status, timing, temperature, reagent locations, and protocol. Protocols are easily shared and modified. **D-E)** Bi-directional flow streams drive reagents to and through specimens for rapid uniform infiltration. **F-G)** Optimized mixing modes for different specimens. *Aspirate-Dispense* **(F)** can repeat every 1/2 second or slower for any number of cycles (control settings). *Aspirate-Mix-Dispense* **(G)** fully immerses specimens then agitates with gentle bidirectional mixing to drive reagent through specimens and then dispenses. Used for multiple specimens per capsule, stacked capsules, and delicate specimens. Carryover volume after dispense **(F or G)** approaches zero and can be further reduced with "blow-out" steps.

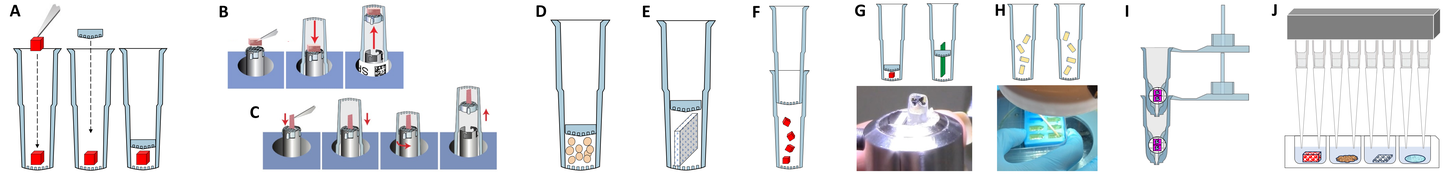

Figure 2: Purpose-built Specimen and Grid Capsules. A) mPrep/s (specimen) capsules entrap tissue, agar-enrobed specimens, pellets, organoids, etc. using mPrep/s screens placed with an insertion tool (not shown) or using mPrep/s Workstation **(B-C)**. Workstation can optionally orient specimens by **B**) entrapping specimen between capsule bottom and screen, or **C**) orienting long specimens (nerve fibers, cell culture substrates) by clamping the back of the specimen. **D**) Entrap several specimens with mPrep/s screen. **E**) Planar specimens cut to capsule diameter (~4 mm) such as cells on coverslips. **F**) Entrap 1 to 8 specimens in each capsule by capping with another capsule. Also used to trap high-pressure frozen specimens/planchets for cryo freeze-substitution [9]. **G)** Specimens entrapped and oriented with screens can be embedded and sectioned in the capsule. **H)** Specimens not screen-entrapped are easily removed to embedding molds. **I**) ASP process TEM grids in mPrep/g capsules, stacked if needed to multiply capacity, used for immunogold labeling [10-11]. **J)** Specimens too large to fit in mPrep/s capsules (larger than ~4 x 9 mm) such as large tissue specimens and entire coverslips are processed in 12-, 24-, or 96-well plates using pipette tips on the ASP head to deliver & agitate reagents.

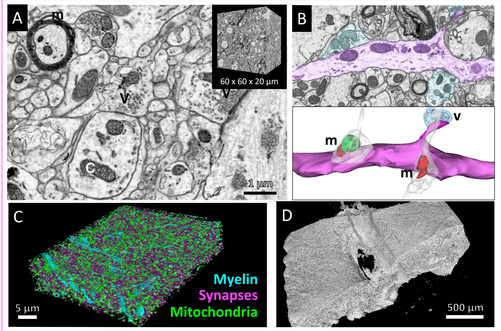

Figure 3: vEM rat brain cortex: ASP-prep in 8 hrs [12-13]: A) Single plane and 3D volume (inset), mitochondria (m), cristae (c), synaptic vesicles (v). B) Dendrite (magenta) single plane shows synapses (red & green), and 3D volume image. C) AI identification & quantification enabled by robotically uniform preparation. D) X-ray microscope image from the same specimen preparation.

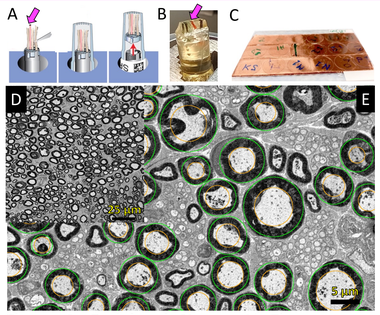

Figure 4: Peripheral Nerve: A) Three nerves oriented in capsule with fiduciary thread (arrow), then ASP-prepared for vEM (Figure 1) and B) resin embedded in mPrep/s capsule (arrow). C) 1 µm sections mounted & UrAc-Pb stained on coverslips, then arrayed on copper tape. D-E) SEM images with auto-segmented axons (E) [8].

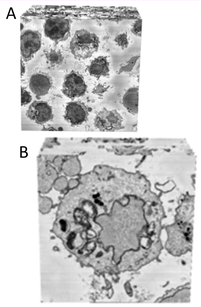

Figure 5: B-Cell Pellets: Splenic mouse B-Cells were pelleted, enrobed in low melting agarose, and ASP processed for vEM.
A) Volume image of pellet, B) Individual B-cell [8,14].

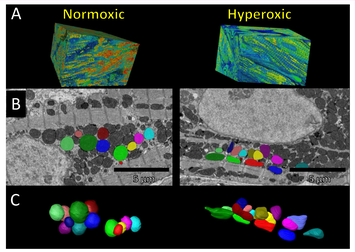

Figure 6: Heart right ventricle from neonate ICU model [15]: vEM of heart right ventricle from control 'normoxic' FiO_2 neonate and 'hyperoxic' ICU rat pups. A) vEM 25 μm^3 cubes. B) Single slice vEMs with segmented mitochondria. C) 3D-rendered mitochondrial shapes differ between normoxic & hyperoxic. ASP prep 7.5 hr before resin curing from perfusion-fixed rats. vEM at ThermoFisher Nanoport, Hillsboro, OR. [16].

Illustrated mPrep Automation Capabilities

mPrep ASPs can prepare nearly any type of specimen. Examples shown include:

- Serial-block-face vEM of rat brain cortex (Figure 3) with artificial intelligence (AI) segmented synapses, myelin, and mitochondria. Fig 3D shows an x-ray microscopy image of epoxy-embedded tissue within a mPrep/s capsule, using the preparative method shown in Figure 2G, illustrating an efficient correlative multi-scale imaging method.
- Multiple peripheral nerves imaged for quantitative AI analysis using a novel method similar to array tomography (Figure 4), prepared using mPrep/s method in Figure 2G.
- vEM imaging of multiple B-cells and an individual B-cell. (Fig. 5), ASP-prep after pelleting & agarose enrobing [8,14].
- Heart ventricle vEM with segmented mitochondria (Figure 6) examines mitochondria in a Neonatal Intensive Care Unit (NICU) model for hyperoxic incubators [15-16].

Some additional specimens not discussed herein include primate brain & cancer models [2,4], renal & muscle biopsies [8], planaria [17], liver, yeast, retina, fish tissues [8,18], and cells on coverslips. Based on these and additional reports, the reduction in specimen prep time and hands-on effort using ASPs is summarized in Figure 7.

Figure 7: Specimen Prep Time & Effort Efficiency. Typical process times and cumulative hands-on effort are shown for manual & ASP prep for TEM & vEM [2-4,8,12-13,15-16].

References

1. LM Collinson et al., Nature Methods 20 (2023) doi.org/10.1038/s41592-023-01861-8.
2. Stempinski ES et al. Methods Cell Bio (2023) 177:1 doi.org/10.1016/bs.mcb.2023.01.009.
3. GJ Kidd and EK Benson, Microsc Microanal 29 (2023) 1083.
4. JL Riesterer et al., Microsc Microanal 29 (2023) 1078.
5. Hsia R (2021) BioEM Technologists Career Survey. BioEM Talks.
6. Hsia R (2015) Microsc Microanal 21(S3):175.
7. Wallrabe H et al. (2014) Microscopy Today, 22(2):36.
8. SL Goodman et al., Microscopy Today, 32:1 (2024) p.16, doi.org/10.1093/mictod/qaad108
9. Bélanger, S, et al. (2022). Front Cell Develop Bio. 10. https://doi.org/10.3389/fcell.2022.933376
10. Marques P et al (2018) Microsc Microanal 24:1300.
11. Lillehoj EP et al. (2019) J Biol Chem. 249(2):662. DOI10.1074/jbc.RA118.006022
12. Benson E et al. (2020) Microsc Microanal 26(2):1372.
13. Goodman SL et al. (2019) Soc. Neuroscience: 429.02.
14. Pal A, et al.. Nutrients 2023, 15, 4807. doi.org/10.3390/nu15224807
15. Tetri LJ et al. (2018). Front Physiol 9:326. doi: 10.3389/fphys.2018.00326
16. Goodman SL et al. (2019) Microsc Microanal, post-deadline poster PDP-46.
17. McLain ML & Nowotarski SH. Methods Cell Biol (2023) 177:9 https://doi.org/10.1016/bs.mcb.2023.01.013
18. Peloggia J et al (2021) Dev Cell, 56(9)1296.https://doi.org/10.1016/j.devcel.2021.03.027