Creating Efficient Workflows for Electron Microscopy Laboratories with Automated Specimen Preparation

Steven L. Goodman,[1,2]* Emily K. Benson,[3] Noah A. Flint,[4] Louis E. Dye,[5] Melissa F. Chimento,[6] Edward Phillips,[6] Jeffrey W. Percival,[1] and Grahame J. Kidd[3]

[1]Microscopy Innovations, LLC, Marshfield, WI
[2]School of Medicine and Public Health, University of Wisconsin, Madison, WI
[3]3DEM Ultrastructural Imaging and Computation Core, Lerner Research Institute, Cleveland Clinic, Cleveland, OH
[4]ARUP Laboratories, Salt Lake City, UT
[5]Microscopy and Imaging Core, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD
[6]High Resolution Imaging Shared Facility, University of Alabama at Birmingham, Birmingham, AL

*Steven.Goodman@MicroscopyInnovations.com

Abstract: Electron microscopy (EM) is essential to the biological and biomedical sciences and clinical diagnostic pathology. Electron microscopy specimen preparation is laborious and time-consuming, with transmission EM (TEM) chemical preparation typically requiring 1–3 days, while volume electron microscopy (vEM) takes 3–5 days of tedious manual reagent exchanges every few minutes or hours. This places a considerable burden on laboratory scientists with the ongoing demand for TEM, and the rapidly growing demand for vEM due to its potential to revolutionize structural biology, connectomics, and related fields. This burden is exacerbated by a shortage of trained electron microscopy scientists as current staff retire, and few enter the workforce. This report provides four case studies to illustrate how automated and faster specimen preparation workflows using mPrep™ Automated Specimen Processors (ASP-1000™ and ASP-2000™, Microscopy Innovations, LLC) free electron microscopy staff in academic and pre-clinical research labs and a clinical pathology laboratory.

Keywords: transmission electron microscopy, volume electron microscopy, workflow, automation, efficiency

Introduction

Electron microscopy (EM) is essential for the biological sciences and clinical pathology. EM laboratories provide services that include transmission electron microscopy (TEM), scanning electron microscopy (SEM), and 3D volume EM (vEM). vEM includes serial block face, array tomography, focused-ion-beam SEM, and serial section TEM [1]. EM labs may also provide immunogold labeling and cryogenic EM, which may include freeze substitution where cryo-prepared specimens are chemically prepared for non-cryogenic EM imaging.

Biological EM began in the middle of the last century with chemical fixatives, stains, and resin embedding, which was recognized with the 1972 Nobel Prize for Physiology or Medicine [2]. This chemical preparative methodology has proven to be extremely robust, and today it remains the most common method for biological research and clinical pathology. In 2023, vEM, which uses the same type of chemical preparation, was identified as "one of the 7 technologies to watch in 2023" in a *Nature* editorial [3] and was described as "a quiet revolution" [1] since it enables a new world of EM discovery with its 3D molecular-resolution imaging in near-millimeter volumes. vEM was also the subject of an inaugural 2023 Gordon Research Conference [4] and a 2023 *Methods in Cell Biology* edition, *Volume Electron Microscopy* [5].

While TEM and vEM microscopes are highly automated, most TEM and vEM bio-specimen preparation remains intensely manual; specimens are sequentially treated by immersion in a multi-day process of aldehyde fixatives, toxic heavy metal stains (for example, osmium tetroxide (OsO_4) or uranyl acetate (UrAc)), solvents, and embedding resins. Manual TEM preparation typically requires 2–3 days [6,7], while vEM preparation takes 4–5 days [8,9], with both requiring tedious manual reagent exchanges every few minutes to hours. While microwave and carousel EM processors can accelerate or automate some TEM workflows, these are not suitable for all specimens and are not capable of vEM preparation, immunolabeling, or post-cryo freeze-substitution vEM [10,11].

The intense hands-on labor for specimen preparation is problematic since it makes poor use of highly trained EM staff, with 81% having MS or PhD degrees [12,13]. Further exacerbating this problem is that we can expect many EM scientists and technologists to retire relatively soon. Since 44% are 51 years or older, there is a limited pipeline of new personnel due to few formal training programs, and 47% of lab managers have never trained a novice [13,14]. Core lab work also demands the highest scientific quality [15] while typically requiring 55 hours/month on administrative tasks, thus it is not surprising that lab managers report difficulty in attracting new staff [12,16].

Automated specimen preparation can free lab personnel from the burden of manual reagent processing, but only if automation can perform the necessary wide range of complex and custom preparation protocols. In 2015, Microscopy Innovations, LLC introduced the mPrep™ ASP-1000 Automated Specimen Processor [17] and, in 2022, introduced the ASP-2000, which adds reagent temperature control and an all-new control Dashboard that enhances the capabilities of both ASP models [18]. Today there are dozens of ASP protocols for TEM, vEM, immunogold labeling, and other applications to meet the needs of today's EM labs by reducing hands-on labor from days to about 1 hour for nearly any type of preparation protocol [18–33]. This can free highly trained EM staff to focus on imaging, analysis, and other knowledge activities while reagent processing is performed automatically.

This report presents four case studies from academic, preclinical, and clinical diagnostic EM labs that illustrate how they use automated specimen preparation workflows to increase

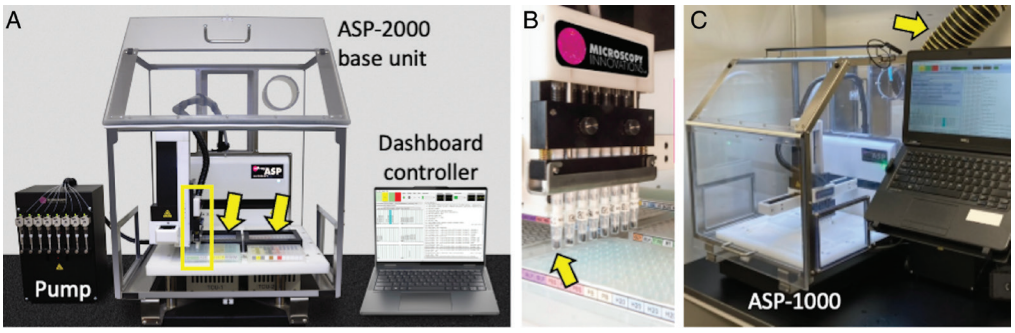

Figure 1: mPrep Automated Specimen Processors: A) ASPs consist of a base unit with an integrated fume enclosure that holds mPrep specimen or grid capsules on an 8-channel pipetting head (box) attached to an X-Y-Z robotic arm, with a pump module and a computer running the ASP Dashboard. With the ASP-2000, as shown, 2 of the 6 microplates located on the deck are temperature-controlled (arrows). B) Reagents are aspirated (pipetted) into mPrep capsules (arrow) attached to the 8-channel head to bathe the specimens with reagents located in any of up to 6 microwell plates on the ASP deck. Reagent delivery, agitation, timing, and temperature (ASP-2000 only) are controlled by the ASP Dashboard. C) An ASP-1000 is installed to vent directly into the house fume system (arrow) with the ASP Dashboard PC mounted above the benchtop.

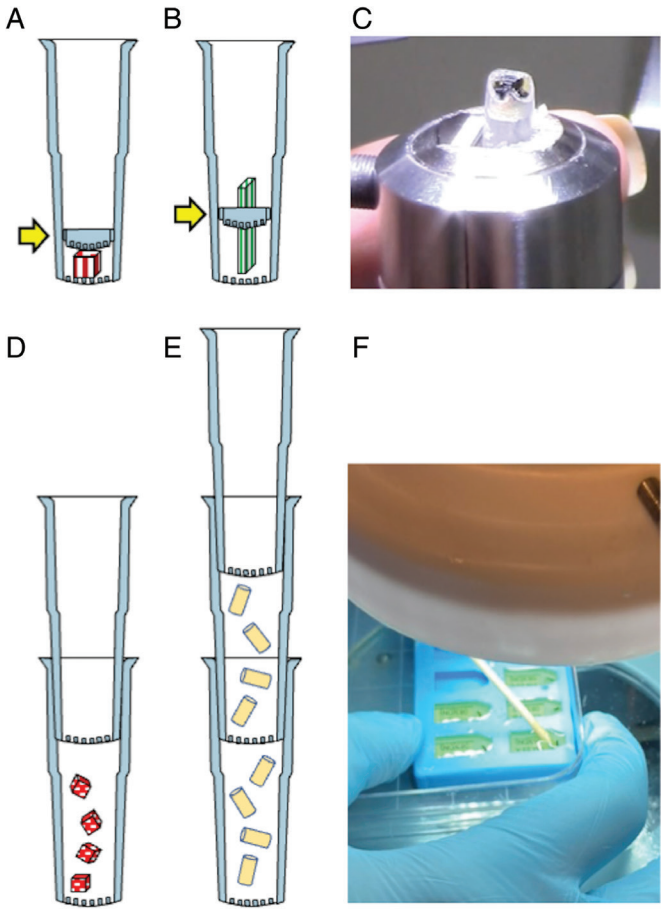

Figure 2: mPrep/s specimen capsule workflows: Specimens can be entrapped in mPrep/s capsules with adjustable mPrep/s screens (arrows) for a desired orientation by gentle "compression" (A) or by pinching the back end of a long specimen (B). Specimens may then be prepared and embedded in the capsule and clamped in a microtome chuck (C) for sectioning of oriented specimens. One or more specimens may be entrapped in mPrep/s capsules (without screens) by capping with a second empty mPrep/s capsule (D), or for greater capacity, two specimen-containing capsules can be stacked (E). After 100% resin infiltration, stacked capsules are separated, and the specimens are removed and embedded in conventional molds (F).

their productivity while reducing reagent consumption and improving reproducibility.

Methods and Materials

ASP instruments. mPrep ASPs (Figure 1) consist of an enclosed base unit where specimens entrapped in mPrep/s capsules (or grids loaded into mPrep/g capsules) are attached to an 8-channel pipetting head (box), and processing reagents are loaded into 12- and 96-well microplates. Under the control of the ASP Dashboard and actuated by a precision pump, reagents are aspirated (drawn into) the capsules to immerse the specimens (or grids). The entire system is composed of solvent and oxidization-resistant materials, including user-replaceable microporous filters, for long-term durability with EM reagents.

The microplates that hold the reagents are selected for the preparation protocol. For example, if all specimens on each shaft of the 8-channel pipette head (Figure 1B) are to receive the same reagent sequence, then 12-channel microplates can be used so that all specimen or grid-containing capsules share reagents from the same microplate reservoir column. By contrast, 96-well plates enable each specimen (or grid) capsule to receive reagents from different reservoirs to enable protocols where individual specimens or grids are prepared simultaneously with different reagents, stains, antibodies, or labeling titrations. Plates can also be covered to cut evaporation or oxidation.

ASPs follow the selected step-by-step protocol from the ASP Dashboard controller by moving the 8-channel head with the attached specimen (or grid) capsules to the specified reagent location, where the pump aspirates (draws in) a reagent into the capsule for the specified time and agitation. The ASP then dispenses this reagent and moves to the next reagent or rinse. ASP-2000s provide protocol-controlled or operator-controlled reagent temperatures from 0–100°C for two microplate positions (Figure 1A, arrows). Figure 1C shows an ASP-1000 installed in ARUP labs for clinical biopsy TEM preparation.

Specimen capsules. mPrep specimen capsules (and grid capsules, not shown) entrap specimens (and grids) to prevent their loss, damage, or misidentification [34]. Figure 2 illustrates several specimen workflows. Specimens can be oriented in capsules prior to embedding, which is facilitated using the mPrep/s Workstation, and then embedded and sectioned within the capsule (Figure 2A–C). Or mPrep/s capsules can entrap several specimens by loading these into one capsule and then trapping them with a second empty "top" capsule. For even greater capacity, two specimen-containing capsules can be stacked and capped with an empty capsule (Figure 2D–E). These are then attached to the ASP pipette head (Figure 1B) for processing through 100% resin infiltration. The stacked capsules are then disassembled, and the specimens are removed for embedding in conventional molds. The

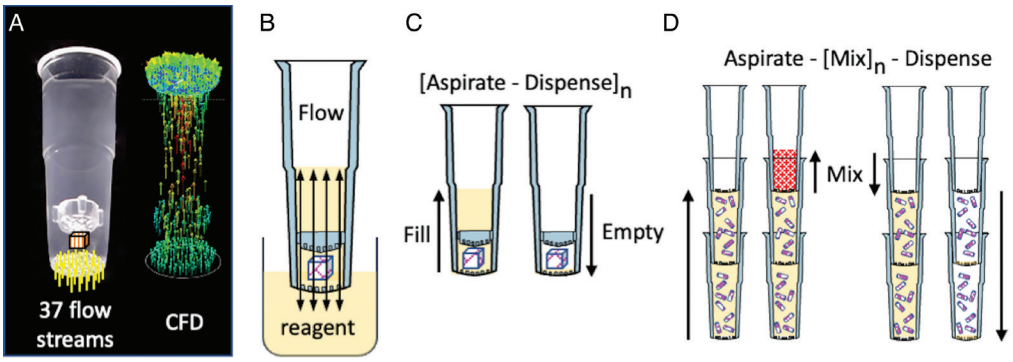

Figure 3: Reagent infiltration: A) Bidirectional reagent flow is directed to and through specimens from 37 parallel flow streams to accelerate infiltration as shown with computational fluid dynamic (CFD) analysis. B) Reagents are delivered and agitated from microplate reservoirs. Reagent agitation is tailored for different specimens by protocol commands: C) Aspirate–Dispense fills and then empties capsules, which can be repeated as fast as every half second for "n" repetitions, at the specified fill-empty rate and volume. D) Aspirate–Mix–Dispense first fills capsules to immerse specimens, then provides "n" mix cycles of gentle bidirectional mixing at a specified volume with specimens remaining immersed, and then empties the capsules before moving to the next reagent.

demonstrated capacity for stacked capsule workflows is up to 8 tissue pieces per capsule for TEM (see ARUP case study below), and up to 4 tissue pieces per capsule for vEM, which provides a capacity of 128 TEM tissue specimens or 32 vEM specimens [28,29].

Reagent delivery. ASPs and mPrep/s capsules provide directed reagent flow (Figure 3A–B) for complete and rapid reagent infiltration. Computational Fluid Dynamic (CFD) analysis shows that the dozens of pores in the mPrep/s capsule bottom provide parallel fluid flow directed to the specimen on in-flow (aspiration) and outflow. Agitation is achieved by repeated aspirate and dispense cycles. Reagents can be dispensed to zero volume for each cycle, or agitation can maintain a volume to keep specimens always immersed, as suitable for multiple loose specimens per capsule, or particularly fragile samples (Figure 3C–D). For single capsules, a typical aspirate volume is 100 µl, with 300-600 cycles as rapidly as every 0.5 to 2 seconds for low-viscosity reactive or active staining reagents such as OsO_4 and UrAc. Fewer cycles, such as 10–50, are suitable for rinses, while slower fill/empty speeds are used for viscous resins. The ASP pump (Figure 1) provides the necessary very wide range of precise flow rates and volumes for reagent agitation and exchanges.

The effective processing volume for each reagent step is determined by the microplate well volume. For 12-well plates, this is 4–5 ml shared by all specimens on the pipetting head, while for 96-well plates this can be as little as ~35 µl per capsule or as much as 1 ml per capsule. With typical 1 mm^3 (1 µl volume) specimens, the reagent-to-specimen volume can range from 35:1 when minimizing consumption of expensive reagents, to 625:1 (5 ml shared by 8 capsules) in 12-channel microplates, and to 1000:1 with deep-well 96-well plates filled with 1 ml/well. These ratios exceed the historically suggested 10:1 ratio of reagent volume to specimen volume [6,7]. After each reagent step, the remaining reagent in the capsule approaches zero. This near-zero carryover enables faster processing since subsequent rinse steps need not remove excessive carryover. This is possible because ASPs will aspirate the next reagent within seconds, thus there is no risk of specimens drying out between reagent steps, unlike manual vial processing.

ASP control interface. The ASP Dashboard Controller was entirely revised in 2022 [18] to simplify reagent agitation commands for high-capacity specimen preparation (Figure 3D), improve ease-of-use, and further reduce reagent carryover. The Dashboard automates virtually any protocol by enabling an unlimited number of protocol steps and reagents with an intuitive interface that mimics manual preparation descriptions: Reagent, Time, Repeats, Agitation, and Temperature (ASP-2000 only). To aid users who wish to modify existing protocols or create new protocols, popup help provides assistance. ASPs also provide alerts (popup messages, sound, SMS text messages) to inform users when, for example, the "Protocol is completed" or it is time to add a fresh, volatile, or labile reagent. Another 2022 Dashboard improvement enables all ASP users to seamlessly share protocols to help the EM community and enhance reproducibility [14,15,18,20].

Results

We describe here how four EM labs use mPrep ASP automated workflows for TEM and vEM specimen preparation. These case studies include research core TEM labs at the USA National Institutes of Health and the University of Alabama Medical Center. The third case study is from ARUP Laboratories, a national clinical reference pathology lab providing TEM biopsy imaging. The fourth is at the Cleveland Clinic's Lerner Research Institute, which does vEM and novel 2D SEM pathology imaging for preclinical studies.

Case Study #1: National Institute of Child Health and Human Development, National Institutes of Health, Microscopy and Imaging Core. The National Institute of Child Health and Human Development, (NICHD) Microscopy and Imaging Core (MIC) is a multi-user microscopy facility providing histology, confocal and super-resolution light microscopy, TEM, and image analysis. MIC resources are provided free of charge to NICHD and Porter Neuroscience Center investigators. TEM is done by a single staff member (coauthor L.E. Dye) in a small, fully equipped 2-room suite that houses a JEOL-1400 TEM with an AMT Biosprint 29 high-resolution camera, and sample preparation equipment that includes a Leica EM UC7 ultramicrotome, a Pelco Biowave, and an mPrep ASP-2000. In a typical year, the lab works on 12 imaging projects that comprise about 320 individual specimens. Most investigators are from the NICHD and the Porter Neuroscience Center, although a few are from other NIH institutes and outside collaborators. Specimens include cell cultures, mouse brains, peripheral nerves, and zebrafish tissues.

Since the ASP-2000 installation in December 2021, nearly all specimens have been prepared with this instrument. Here

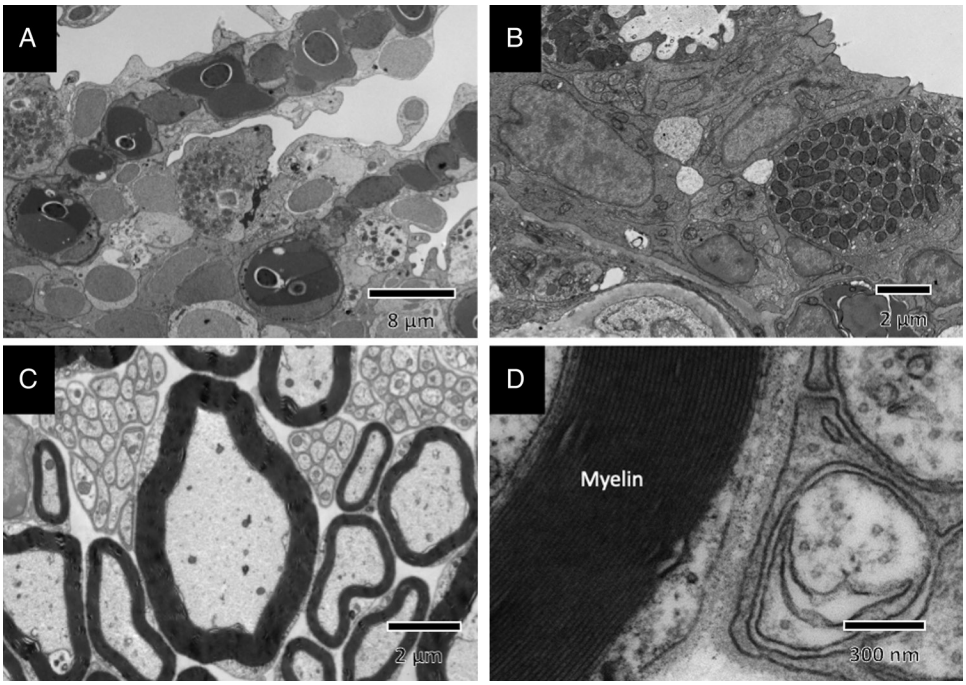

Figure 4: Research TEM applications from the National Institute for Child Health and Development of the National Institutes of Health. A–B) Zebrafish gills, secondary lamellae (or filament), at low and high resolution, prepared with an ASP-2000 process time of 3 hours. C–D) Mouse wild-type sciatic nerve at lower and higher resolution, prepared with an ASP-2000 process time of 45 minutes. Note the uniform infiltration and complete OsO_4 staining in all images, including thick myelin, with a very short process time.

we show two very different specimens. Figures 4A and 4B are of the secondary lamellae (or filament) gill dissected from an adult zebrafish. This specimen was ASP-processed after overnight or longer fixation in 4% glutaraldehyde (GA) in cacodylate buffer. Specimens were loaded with one specimen entrapped per mPrep/s capsule as illustrated in Figure 2A, albeit without a specific orientation. The ASP used the agitation mode diagrammed in Figure 3C, with a protocol of 3 cacodylate buffer rinses, then 2% buffered OsO_4 for 300 aspirate-dispense cycles, 3 water rinses, graded ethanols, acetone, and graded resin infiltration using EMbed-812 medium hard. The ASP protocol slowly warmed the resin to 40°C to facilitate resin infiltration by reducing its viscosity. The total ASP process time was 3 hrs. Fully infiltrated specimens were then removed from the capsules and oriented for cross sections in Chien molds, with the resin then cured overnight at 60°C. Sections on grids were stained with UranyLess and lead citrate and imaged with a JEOL-1400 TEM at 80 kV. Note that the OsO_4 post-fixation staining, and resin infiltration are complete even with just a 3-hour protocol.

The second example is of a mouse wild-type sciatic nerve (Figures 4C and 4D). This specimen was perfusion-fixed with 4% paraformaldehyde (PFA) and 2.5% GA in phosphate-buffered saline. The nerves were then entrapped in mPrep/s capsules with screens without orientation, with one sample per capsule. The reagent sequence was the same as with the gill specimens, including resin warming, however here the ASP protocol used very rapid agitation using the mode diagrammed in Figure 3C. With this rapid agitation, the total process time from aldehyde rinse-out through 100% epoxy infiltration was just 45 minutes. This illustrates the potential of very fast ASP process speeds, although this may not be suitable for more fragile specimens. Embedding, section staining, and imaging were the same as described for the gill specimens. Here, also note that the OsO_4 post-fixation staining of the myelin membranes and resin infiltration are complete even with this very fast 45-minute protocol.

Case Study #2: University of Alabama-Birmingham High-Resolution Imaging Facility. The mission of the High-Resolution Imaging Facility (HRIF) is to provide state-of-the-art imaging resources, training, and technical support for the University of Alabama at Birmingham (UAB) research community. The HRIF provides room-temperature TEM, confocal, super-resolution, live cell, multi-photon, widefield, and other optical microscopy systems and services. This report addresses only TEM imaging with specimens prepared using chemical preparation methods. The HRIF facility was equipped with a Tecnai Spirit 120 kV TEM (FEI-Thermo Fisher, Hillsboro, OR) with an AMT BioSprint 29 camera (AMT Imaging, Inc., Woburn, MA) for this described TEM work. Preparative equipment includes a PELCO Biowave Pro, two Leica UC7 ultramicrotomes, a Leica EM-TP automated tissue processor, and two ASP-2000s.

There are four lab personnel, with two providing TEM services. HRIF staff provide support and services for over 250 UAB labs from multiple departments for all types of biological and materials specimens from research animals, biopsies, plants, and cell and tissue culture. External academic and external commercial projects are also accepted. Historically, when not disrupted by a worldwide pandemic or by the catastrophic flood that immersed the entire lab in over 3 feet of water in December 2022, the lab would handle between 40 and 50 EM projects annually, with more than 500 samples that required TEM preparation. The lab has recently moved to a new location and will resume full operations in January 2024 with a new JEOL 1400 TEM. Since April 2022, when their first ASP-2000 was installed, the lab routinely prepares specimens with their two ASP-2000s.

Figure 5 shows two examples that illustrate some of the diversity of specimens prepared by HRIF staff (imaged by Kelley Bradley at UAB, from the Robert Kesterson Lab now at Louisiana State University). These are mouse pancreas (Figure 5A) and mouse facial skin (Figure 5B), both from 1-year-old mice, with the facial skin from a neurofibromatosis model Balb6 male. Both mouse specimens were immersion-fixed in 2.5% PFA and 2.0% GA in 0.1 M cacodylate buffer, pH 7.2. Both samples were loaded and processed in mPrep/s capsules, similar to the Figure 3B diagram, however, two specimens were inserted per capsule, separated from each other with a second screen.

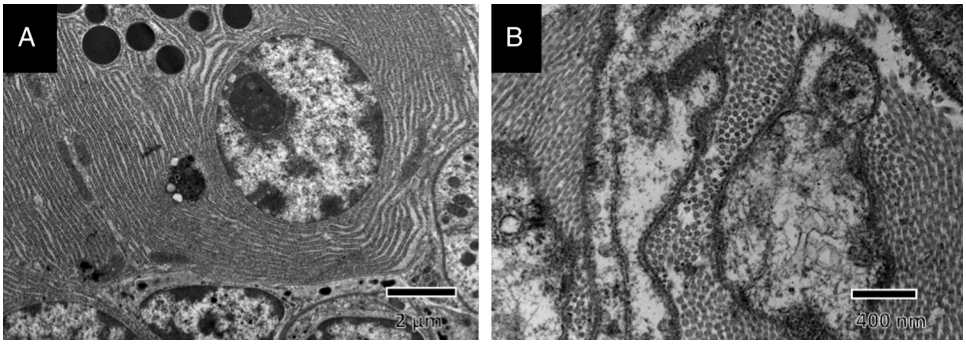

Figure 5: Research TEM applications from the University of Alabama, Birmingham. A) Pancreas from a 1-year-old mouse. B) Facial skin from a 1-year-old Balb6 male neurofibromatosis model mouse. Both were ASP-2000 processed in 4 hours. Note the uniform infiltration and complete OsO_4 staining in both images.

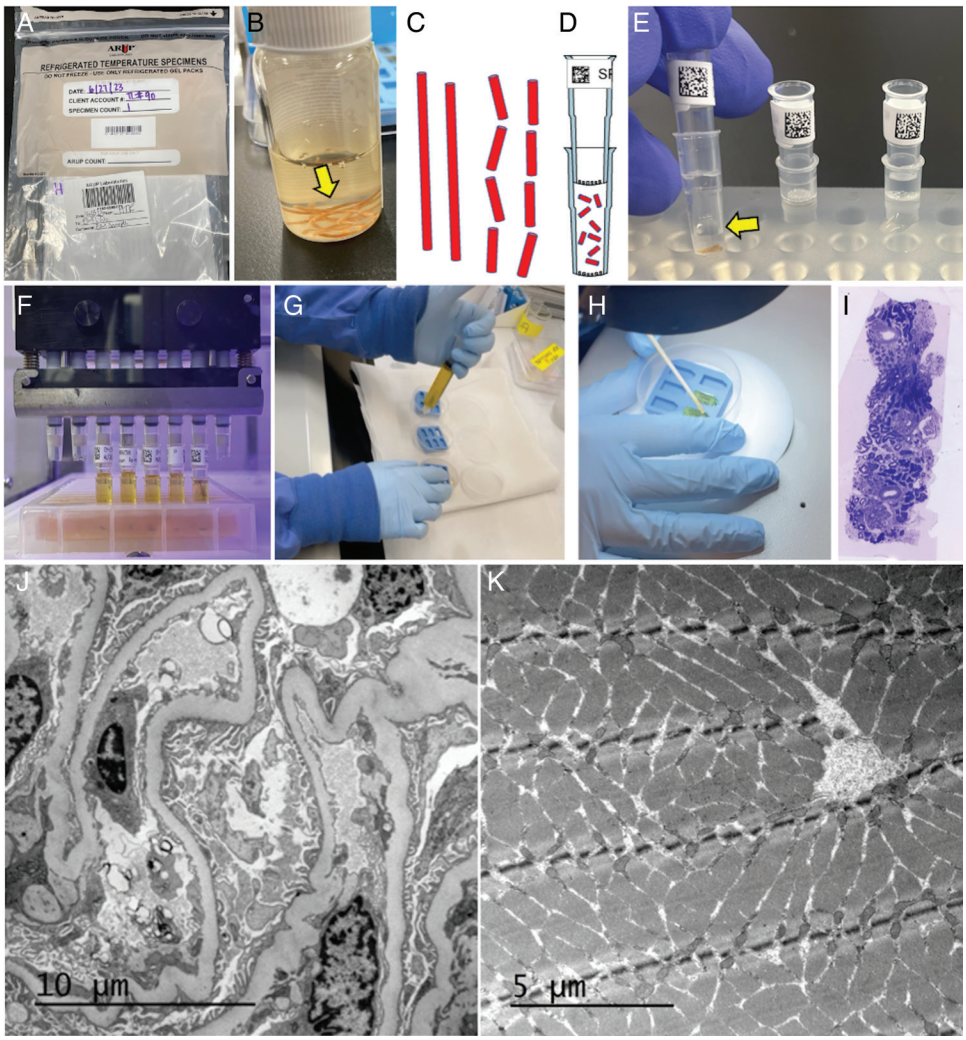

Figure 6: Clinical TEM workflow from ARUP Labs: Renal core (or muscle) biopsies are received in labeled packages (A) containing patient identification barcodes with cores immersed in GA-PA (B). The cores are cut to 1–3 mm lengths (C) with up to 8 of these then placed into each mPrep/s capsule, which is capped with a second barcode-labeled capsule (D). During capsule loading, the capsules are held in a silicone 96-well plate (E) that seals the capsule bottoms to retain GA-PA (mPrep/bench, Microscopy Innovations), with additional GA-PA added as needed to ensure specimens remain immersed (arrow). F) Five specimen-containing mPrep/s capsules on the ASP pipette head after 100% resin infiltration. G–H) Flat embedding molds are filled and then loaded with specimens prior to resin curing. Note that one mold is used per patient. I) Renal biopsy light microscope image used to find regions of interest. J) Renal TEM biopsy image and K) muscle TEM biopsy image.

The specimens were then ASP-2000 processed through 3 buffer rinses, OsO_4, aqueous UrAc, water rinses, serial ethanols, acetones, and finally EMbed 812 epoxy resin using the agitation procedure shown in Figure 3C, and resin warming. The ASP protocol from aldehyde rinse-out through 100% resin infiltration was completed in 4 hours. The resin-infiltrated specimens were removed from the capsules, flat-embedded, and cured overnight at 60°C. Grids were poststained with lead citrate and 1% ethanolic UrAc for TEM imaging at 80 kV. Both specimens demonstrate complete embedding and high-contrast uniform organelle staining with this 4-hour ASP protocol.

Case Study #3: ARUP Laboratories, Salt Lake City, Utah. ARUP Laboratories (Associated Regional and University Pathologists) is a national reference CAP, ISO-15189-, and CLIA-certified diagnostic lab with over 35 years of experience. ARUP is one of the USA's largest clinical pathology labs. Its EM unit prepares over 25 patient clinical renal biopsy specimens every week, for a typical total of 1300 patient specimens per year, plus a variable number of skeletal and heart muscle biopsies.

Every morning, 4–15 renal patient specimens are received at the EM lab in barcode-labeled vials (Figure 6A). Each of these patient specimens consists of one or more 18G needle biopsy cores that have been immersed overnight in cacodylate-buffered GA-PA. The EM staff removes these needle cores from the vials and cuts them into 2-3 mm long segments (Figures 6 B and 6C). Up to 8 of these segments are then loaded into a single mPrep/s specimen capsule and capped in place with a second barcode-labeled capsule to identify the patient (Figures 6D and 6E). The specimens are loaded into the capsules while they are held in a silicone 96-well plate, which provides a liquid-tight seal for the capsule bottom (mPrep/bench, Microscopy Innovations). GA-PA is added as needed to ensure specimens remain immersed (Figure 6E).

The specimen-containing capsules are then attached to the ASP-1000 (Figure 1C) pipette head in one

of two ways, depending on the number of patients in the day. With 8 or fewer patient specimens, single stacked capsules are placed on the pipette head (Figure 2D and Figure 6D–F). As shown in Figure 6F, each pipette channel is actuated independently so the ASP can process any number of its 8 channels at a time. When there are 9 to 16 patient specimens, the capsules are double stacked (Figure 2E) for greater capacity. Thus, as many as 16 capsules, for up to 16 patients, for a total of up to 128 tissue pieces, can be processed at the same time (16 capsules × 8 specimens per capsule).

The ASP protocol duration for 8 or fewer patients/capsules takes 3 hours while the protocol for 9–16 patients takes 3.5 hours, with the longer time due to the longer filling and emptying times with the taller double-stacked capsules. Figure 6F shows the ASP loaded with 5 patient capsules. The ASP protocol is conservative, consisting of 3 buffered GA-PA rinse-outs, OsO_4, aqueous UrAc, 3 water rinses, graded ethanol dehydration (50, 70, 95%), 6 × 100% ethanol, 6 acetone steps, and then graded epoxy-acetone infiltration with epoxy 812 formulation resin of 1:1 and 3:1, and then 3 steps with 100% epoxy. The entire sequence uses 12-well plates filled with 4.5 ml for aqueous reagents and 100% epoxy, and 5.5 ml for acetone and ethanol. To accelerate time to completion, the operator starts the ASP protocol which begins with the 3 GA-PA buffer rinse-outs, and then while the ASP executes these steps, adds the OsO_4, UrAc, and water rinses. The ASP then pauses mid-protocol and alerts the lab for the timely addition of acetone and epoxy resin, but otherwise operates without intervention. At the protocol conclusion, the ASP provides a "completed" alert. A staff member then removes the resin-infiltrated and patient-labeled capsules from the ASP. To ensure no patient mix-ups, each barcode-labeled capsule is placed in a separate petri dish that contains a flat embedding mold with the same number of wells as there are biopsy segments for each patient. The biopsy segments are then removed from the patient's capsule and orientated in patient-labeled resin-filled molds. Blocks are then polymerized overnight at 70°C.

The next morning, block facing, microtomy, semi-thin optical sectioning, thin TEM sectioning, grid staining with UrAc and lead citrate, and TEM imaging are done. Every patient block is sampled with 0.4 μm semithin sections that are toluidine blue-stained on barcoded glass slides (Figure 6I). Then, 3 grids are prepared with a minimum of 2 sections per grid. Imaging is performed using a JEOL 1400 Flash TEM equipped with a Gatan Rio Camera, with images stored on a server where pathologists and clients access them as jpg files for efficient data transfer. The total turnaround time from receipt of the sample to images being available on the server is approximately 24 hours. Figures 6J and 6K provide examples of renal and muscle biopsy TEM images, as used for clinical diagnoses.

Any time a specimen is moved from one container to another, or new labels are used, two persons are involved to ensure no mix-ups with barcode integration in a Lab Information System (LIS). Specimen identities are barcode-tracked except for human-readable labels embedded in epoxy blocks. Two-person ID confirmation assures identity when specimens are transferred from the sample vial into mPrep/s capsules, into embedding molds, for thick sections on barcode-labeled light microscope slides, and to identify the TEM grid box in the LIS grid log system.

This ASP-implemented workflow requires only 1 person-hour of hands-on effort, divided between 2 persons at ~30 minutes each, timed from when EM processing begins with specimen unloading from the as-received vial until the specimens are transferred into flat molds for resin curing (Figures 6F through 6H). This is a substantial person-effort reduction compared to ARUP's prior microwave processing workflow that required 6–7 person-hours, nearly nonstop manual reagent exchanges for microwave processing, and considerable care to ensure specimens were not accidentally pipetted, damaged, or lost during each reagent exchange. Reproducible microwave process timing was especially difficult to achieve on days with higher numbers of specimens, and microwave processing consumed much more reagent.

Case Study #4: Cleveland Clinic 3DEM Ultrastructural Imaging and Computation Core. The 3DEM (Three-Dimensional EM) Ultrastructural Imaging and Computation Core in the Lerner Research Institute (LRI) of the Cleveland Clinic provides state-of-the-art large-volume vEM, including project development, specimen processing, imaging, and image processing and analysis using robust Linux software, open-source applications (ImageJ, Python/TensorFlow, R), and parallelized analysis for high-throughput and deep-learning applications on the LRI Computing Services cluster.

The 3DEM Core specializes in automated SEM approaches. Two use cases predominate, which are serial blockface SEM (SBF-SEM or 3DEM) and array tomography using single 1 μm sections (2DEM section scanning). The 3DEM Core was established three years ago by an amalgamation of the LRI Department of Neurosciences vEM system and Renovo Neural Inc.'s 3DEM Unit, which provides a growing number of users from the LRI and other academic and commercial organizations with expertise and imaging. While the core staff prepare, image, and analyze most samples, training is also provided for interested individuals. The LRI has a separate core to provide TEM, light microscopy, and materials SEM for research and pathology.

Two staff members operate three instruments that are each capable of vEM, slide scanning, and conventional and low-vacuum SEM. One is a Teneo VolumeScope 2 (Thermo Fisher) equipped with the VolumeScope stage-mounted ultramicrotome and MAPS software to facilitate smart-feature operation, and multi-energy deconvolution for virtual slicing, and to support correlative image set overlays. The second two are Zeiss Sigma VP systems equipped with Gatan 3View ultramicrotome stages. Both vEM types are equipped with backscattered electron detectors optimized for low-kV serial block-face SEM, and all systems feature optional low-vacuum modes for samples with exceptionally low conductivity.

With only 2 personnel, most aspects of their work must be automated, and efficient and consistent specimen preparation is paramount. In a typical month, 60 specimens are prepared for vEM imaging, with the goal to keep all three vEM instruments operating at capacity. Some studies date back ten years or more, and samples produced today need to be of the same high quality that they were at the beginning when more staff time was available each day to ensure manual staining consistency. Thus, the 3DEM Core makes extensive use of its ASP-1000 to provide both speed and consistency [19,22,23].

Table 1: Reagent protocol, exchanges, and incubation times for reagent exchanges for vEM manual versus ASP-1000 comparison. *30/30/30 indicates 30 exchanges in 3 different reagent wells.

	Manual benchtop		**Automated ASP-1000**	
Reagent	**Exchanges**	**Time (min)**	**Exchanges**	**Time (min)**
GA-PA fix	Perfuse	Store 5°C	Perfuse	Store 5°C
Buffer rinse	6	30	30/30/30*	3
Tannic acid (some)	1	15	450	15
OsO_4-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% OsO_4	1	180	900	30
Water	5	25	90/90/90/90/90	15
2% UrAc	1	1200	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	1	45
Resin cure 60°C	Into 60°C	2 days	Into 60°C	Overnight
Time	**4 elapsed days**		**1 elapsed day**	
Effort	**2 days work**		**1 hour work**	

Prior to employing ASP-1000 automated preparation, studies were undertaken to compare the quality between manual and ASP specimen preparation using similar brain cortex samples from one GA-PA perfusion-fixed rat. The reagent sequences were identical for both (Table 1), but the manual process required 4 elapsed days of bench processing, while the ASP process was completed in less than 8 hours [19,22]. As shown in Figure 7, 3D perspective projections were similar, while measurements of axon size and myelin thickness were statistically identical (Figure 7A–7D). Further, the overall preparation quality was judged comparable for staining, infiltration, and SBF-SEM sectioning [19,22]. The 3DEM Core workflow now routinely uses the ASP-1000 for preparation due to its repeatability, speed, and much lower effort, and since its consistency provides reproducibility for feature identification and artificial intelligence segmentation (Figure 7E–7H). Additional ASP processing methods have been developed for cell pellet sample preparation, as shown with lymphocytes that were pelleted, enrobed in low-melting agarose, and then processed like tissue pieces (Figure 7I) [31].

For preclinical studies investigating myelination of peripheral nerves, a very different methodology was used to prepare specimens. 2D EM section scanning was used to image nerve cross sections for automated determination of axon diameters, myelin thickness, and axon integrity/pathology. A high-throughput approach was developed using the mPrep/s capsule specimen loading and embedding method shown in Figures 2B and 2C: three segments of aldehyde-fixed peripheral nerve were mounted in one mPrep/s capsule along with a fiduciary-colored cotton thread. These were then ASP processed with 1% OsO_4, graded ethanols, 50:50 propylene oxide, resin, and 100% resin (Figures 8A and 8B). Blocks were cured and sectioned (0.5–1 μm), with the sections mounted on 12 mm coverslips and batch stained with methanolic UrAc and lead citrate TEM stains, which only impregnate a thin surface layer, which is optimal for this method. These were then imaged with a Sigma VP (Gatan) or Teneo SEM (Thermo Fisher) with low-kV backscattered electron detectors at low vacuum. The highest potential resolution was ~4 nm/pixel with 30 nm "optical" sections (imaging at 2 kV), although for most nerve histology studies resolution was 10–40 nm/pixel. Sections were scanned as 4k or 6k pixel tiles using SEM control software. This image acquisition can be scripted using Gatan Digital Micrograph software. Thirty or more samples can be set up for batch imaging, using preset 3-point focusing (Zeiss/Gatan) or automated focusing (Thermo Fisher). At 20 nm/pixel resolution, each section of mouse nerve takes about 1 hour to collect. Processing and analysis can be undertaken using many commercial and open-source software packages. Frequent analyses can be expedited by scripting. This provides a rapid way to image a very large number of axons, from multiple experimental conditions to quantitively assess pathology (Figures 8C and 8D).

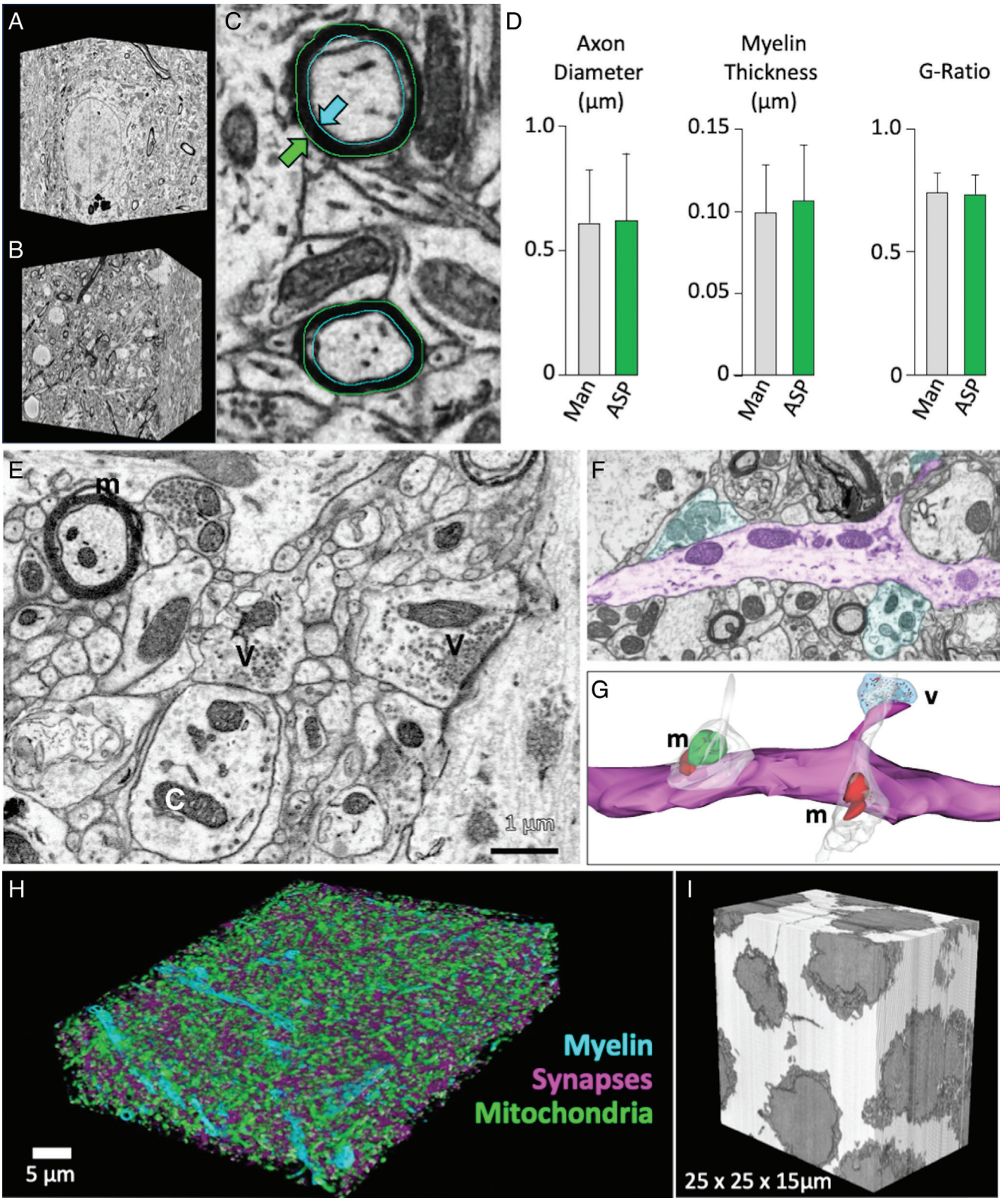

Figure 7: Research vEM from the Cleveland Clinic 3DEM Ultrastructural Imaging and Computation Core. Volume images (60 × 60 × 20 µm) of the same GA-PA perfusion-fixed rat brain cortex that was prepared manually (A) and with ASP-1000 (B). C) Single-plane image showing automated measurement of axon and myelin diameter and thickness, done on both manual and ASP-prepared specimens. D) Bar chart with mean ± SD shows statistically equivalent measures from 4,894 measurements on both specimens. E) Single plane from ASP-prepared specimen shows mitochondria (m), cristae (c), and synaptic vesicles (v). F) Dendrite (magenta) of another single plane image shows synapses (red and green), and in 3D volume image (G). H) Artificial intelligence identification and quantification of myelin, synapses, and mitochondria from another ASP-prepared brain specimen. I) Lymphocyte vEM: lymphocytes were pelleted, enrobed in low-melting agarose, and then ASP-processed for vEM.

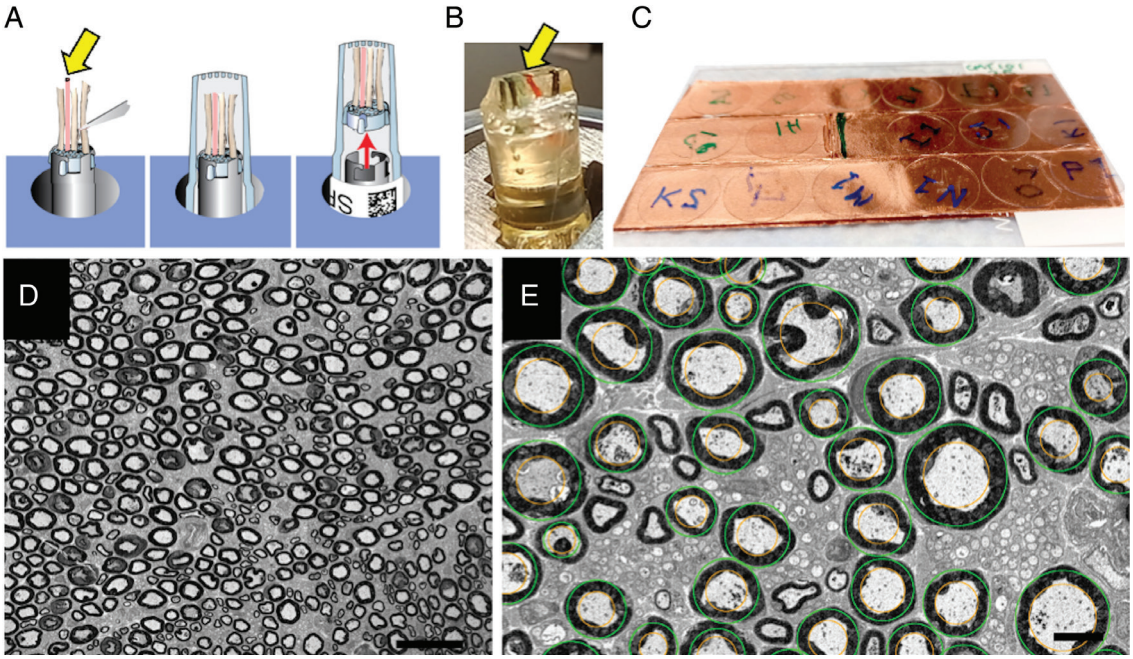

Figure 8: Preclinical research vEM also from the Cleveland Clinic 3DEM lab: A) Diagrammed preparation of 3 peripheral nerves being oriented in mPrep/s capsule with a red fiduciary thread (arrow) prior to ASP postfixing and embedding. B) Photo of 3 embedded nerves and red fiduciary thread (arrow) embedded in epoxy block ready for sectioning (arrow). C) 1 μm sections mounted and UrAc- and lead-stained on coverslips, then arrayed on copper tape. D–E) SEM images with auto-segmented axons for measurement of axon and myelin diameter and thickness.

Discussion

These four case studies illustrate how varied labs use automated TEM and vEM specimen preparation to free staff from repetitive reagent processing so they can focus on imaging, image analysis, client support, and other non-automatable tasks. These case studies also illustrate some of the diversity of specimen types that can be automatically prepared for TEM and vEM, including zebrafish tissues, brain, skin, pancreas, muscle, kidney, and lymphocytes. These and other labs have also used ASPs for many additional EM processing applications, including skeletal and cardiac muscles, breast cancer tumors, planaria, liver, yeast, ocular tissues, agar-enrobed cells and small specimens, acrylic embedding, and both pre-embedding (*en bloc*) and on-grid (post-embedding) immuno-gold labeling [12,18,19,22–24,26–33,35,36].

In addition to the breadth of specimen types, it is also beneficial that ASP automation can prepare specimens much more quickly than manual preparation, as demonstrated in the presented case studies with TEM preparation from aldehyde rinse-out to 100% epoxy infiltration prior to resin curing in just 1–4 hours, and vEM preparation in 6–8 hours, in contrast to typical manual TEM preparation taking 1–3 days [6,7] and 4 or more days for vEM [8,9]. These TEM preparation speeds are comparable to microwave processing [37,38], but as described in Case Study #3, ASP preparation requires substantially less hands-on effort and uses less reagent. In addition, ASPs can also perform vEM, immunolabeling, and other complex protocols.

Of course, fast preparation is valueless if quality is lacking. The quality of ASP versus manual preparation has been investigated in several studies, as partially shown in Case Study #4 (Figure 7), and in additional work from the Cleveland Clinic 3DEM Core [19,22,23]. Stempinski et al. also compared the ASP-2000 to manual preparation in two reports [28,29], with one examining marmoset brains and a murine breast cancer model with a 7-hour ASP preparation compared to 2.5-day manual preparation, for specimens imaged with 3 vEM modes (SBF-SEM, array tomography, and focused ion beam SEM) that included deep-learning quantitative analyses. In all of these comparative reports, ASP preparation quality was at least equivalent to manual preparation. ASP preparation has also been used for vEM studies of planaria [30], zebrafish lateral lines [36], and clinical cancer specimens [32]. Recent reports of improved methods for vEM preparation using high-pressure freezing and freeze-substitution describe using mPrep/s capsule stacking (Figure 2D) as beneficial since the capsules provide efficient trapping of high-pressure frozen specimens at cryogenic temperatures while keeping specimens fully immersed throughout freeze-substitution [10,11]. While these reports performed the freeze-substitution manually, an ASP-2000 could have automated the freeze-substitution vEM preparation steps above 0°C.

The number of specimens that need to be prepared in EM labs can vary greatly, and ideally, preparation should be both labor and reagent-efficient, especially with expensive and toxic chemicals. As shown here, ASPs can simultaneously prepare up to 128 tissue pieces (Case Study #3), or 16 tissue samples (Case Study #2), or 24 nerves (Case Study #4) while using only 4–5 ml at each reagent step. Up to 32 vEM specimens have also been prepared simultaneously by placing 4 tissue pieces per capsule, as diagrammed in Figure 2D [28,29,32]. As illustrated in Figure 6F, ASPs can process with fewer than 8 attached capsules at a time, thus enabling

automated consistency for even just one specimen. ASPs can also minimize reagent consumption to as little as 35 µl for small tissue specimens or TEM grids when desired with high-cost and toxic reagents. Besides saving money, low reagent consumption in combination with the minimal handling provided by automated preparation can reduce potential staff exposure to hazardous reagents [28,29].

Summary

mPrep Automated Specimen Processors and mPrep capsules are adaptable to a wide range of EM preparation applications for research and clinical TEM, vEM, and related types of microscopy. As illustrated in these case studies, the ASP processor and capsule system is adaptable to a wide range of lab needs by providing rapid, reproducible, and both labor and reagent-efficient preparation. With the recent major update to the ASP Dashboard, the ability to process larger numbers of specimens is enhanced and simplified, along with new control options for more efficient reagent handling to speed processing with robust and fragile specimens. The new ASP Dashboard Controller also enables any ASP-equipped lab to easily share precise preparation protocols with any other ASP-equipped lab [18,20,21], which can help the EM community improve research quality and enhance productivity even with the growing demand for laborious vEM in busy and understaffed labs [1,5,13–15].

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