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Automated Volume Electron Microscopy for Neuroscience

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Abstract:

Volume Electron Microscopy (vEM) with its 100 um^3 and larger datasets is ideal for neuroscience. vEM includes serial block-face scanning EM (SBEM), FIB-SEM, and array tomography. While vEM imaging is automated, manual specimen prep of neurological specimens is a 4-5-day process of tedious error-prone reagent exchanges (fixatives, labels, stains, solvents, resins) that may not provide the consistency required for artificial intelligence image segmentation (AI) and quantification that is practically essential. The 3DEM Core provides state-of-the-art vEM project development, specimen prep, imaging, and analysis using open-source (e.g. Image J, Python/TensorFlow) and parallelized analysis for high throughput and Deep-Learning applications. The Core specializes in automated SBEM imaging and analysis of brain, neural tissues, organoids, cell pellets, and cell cultures. The Core automates specimen prep with the mPrep ASP-1000 (ASP, Automated Specimen Processor) to provide repeatability, speed, minimize reagent consumption, cut hands-on effort, and provide reproducibility for reliable feature identification and AI segmentation. ASP prep quality was validated by comparing cortex from the same prefusion-fixed rat brain prepared in a 4-day manual process to an 8-hr ASP-prep. Manual and ASP specimens yielded comparable 3D perspective projections, staining, infiltration, sectioning, and statistically identical measures of axon sizes, myelin thickness, and G-ratios. The Core has developed automated AI image analyses including non-linear 3D measures of internodal myelination of large-diameter motor axons, and an assay of peripheral nerve axon diameters, myelin thickness, and integrity/pathology using 2D section scanning: 3 peripheral nerve segments (different experimental conditions) are mounted in a mPrep/s capsule with a fiduciary thread. These are then vEM-prepared and embedded in the capsule. Semithin sections on coverslips are then Ur and Pb batch stained and imaged with a Sigma VP or Teneo SEM with low kV backscattered electron detectors (Gatan, ThermoFisher respectively). Scripted imaging (Gatan Digital Micrograph) enables 30+ samples to be imaged at 20 nm/pixel in 1 hour. This provides automated quantitative axon pathology from multiple experimental conditions to assess therapeutics. The ASP also enables vEM prep of in vitro 2D cell cultures, 3D organoids, and cell pellets. With ASP processing, the 3DEM core now employs a 3-step automated workflow comprising specimen prep, imaging, and concluding with deep-learning AI analysis. With this automated workflow, Core scientists now can focus on human-only knowledge tasks.

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Introduction

Volume Electron Microscopy (vEM) with its 100 um^3 and larger datasets is ideal for neuroscience. vEM includes serial block-face scanning EM (SBEM), FIB-SEM, and array tomography. While vEM imaging is extensively automated, manual specimen prep of neurological specimens is a 4-5-day process of tedious error-prone reagent exchanges that may not provide the consistency required for artificial intelligence image segmentation (AI) and quantification that is essential.

The 3DEM Ultrastructural Imaging & Computation Core specializes in automated SBEM imaging and analysis of brain, neural tissues, organoids, cell pellets, and cell cultures. The core provides state-of-the-art vEM project development, specimen prep, imaging, and analysis.

Methods

The 3DEM Core uses an automated workflow for neural tissues that provides speed, labor efficiency, reproducibility, and high quality. This includes automated tissue/specimen preparation, SBEM (and array tomography) imaging, analysis using robust Linux software with open-source applications (ImageJ, Python/TensorFlow, R, Dragonfly), and parallelized analysis for high throughput and Deep-Learning applications [1]. Instruments include a Microscopy Innovations mPrep ASP-1000 automated specimen processor, a ThermoFisher Teneo VolumeScope 2, and two Zeiss Sigma VP SEMs with Gatan 3View ultramicrotome stages. All SEMs are equipped with low kV backscattered electron detectors and low-vacuum modes for low-conductivity samples.

Automated Specimen Preparation

Specimen preparation at the 3DEM Core uses the mPrep ASP-1000 automated workflow (Fig. 1) with mPrep/s specimen capsules (Fig. 2). The ASP enables nearly any protocol for different specimen types and applications, and the processing of just 1 or up to 128 specimens at a time. Figure 3 shows how ASPs use parallel microfluidic directed reagent flow for rapid reagent infiltration and efficient reagent exchanges [2].

Results and Discussion

Brain vEM with ASP preparation

ASP prep quality was validated by comparing cortex from a glutaraldehyde-paraformaldehyde (GA-PA) prefusion-fixed rat brain. Parallel samples were prepared with a standard 4-day manual bench process and with an 8-hr automated ASP-1000 protocol (Fig. 4A) using the same reagent sequence: buffer rinses, tannic acid (some blocks), OsO_4-KFeCN, TCH, OsO_4, UrAc, Pb, graded ethanols, acetone, graded epoxy until 100% epoxy infiltrated and ready for 60C oven curing.

Manual and ASP-prepared specimens yielded comparable 3D perspective projections, staining, infiltration, sectioning, and statistically identical measures of axon sizes, myelin thickness, and G-ratios (Fig. 4B) [3-4]. The reproducibility of ASP-prep enables facile AI segmentation of synapses, mitochondria, myelin, etc. ASP blocks from this prep can also be imaged with X-ray microscopy for correlative imaging of larger 3D volumes (Fig. 4C-F). The quality and consistency of ASP-prep has also been reported in other comparisons of ASP and manual prep of planaria [5], brain, and tumor tissues [6-7].

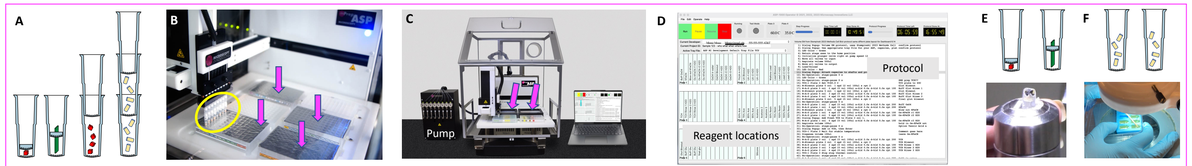

Figure 1: mPrep™ System Workflows: ASP™-1000s and ASP-2000s reduce personnel effort and can perform nearly any vEM or TEM protocol: A) Specimens are loaded into mPrep/s capsules. Can orient in individual capsules, or load 1-8 specimens per capsule for high capacity. B) Capsules are attached to an 8-channel ASP™-1000 head (circled). Each of the 8 channels can deliver different reagents. Capsules can be stacked on each pipettor shaft to multiply capacity, enabling simultaneous preparation of up to 32 tissue specimens for vEM and up to 128 for TEM [2]. 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 microplates. 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.

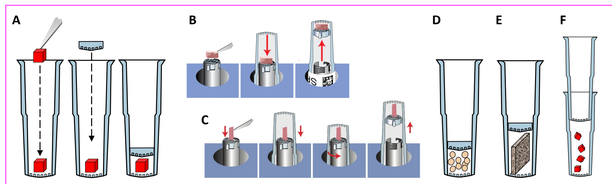

Figure 2: Specimens in mPrep/s capsules: Capsules are used differently for different workflows. A) Tissue specimens, pellets, etc. are placed in capsules and entrapped with mPrep/s screens using an insertion tool (not shown). B) The mPrep Workstation can orient specimens by entrapping between capsule bottom and screen, or C) orient long specimens (e.g. nerves) 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 the screen. Capsule pores = 300 µm. Enrobe small specimens (e.g. agar) to entrap in capsules.

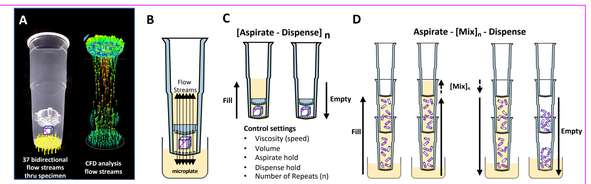

Figure 3: Rapid Reagent Infiltration: A) Bidirectional flow directed to and thru specimens from 37 parallel flow streams accelerates infiltration. B) Reagent is delivered from 12 or 96-well microplates. Agitation is tailored for application, specimen robustness, and specimen numbers: C) ***Aspirate – Dispense*** fills and then empties capsules. Can repeat every ½ sec 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 (control settings), then dispenses. Carryover volume after dispense approaches zero further accelerating process speed.

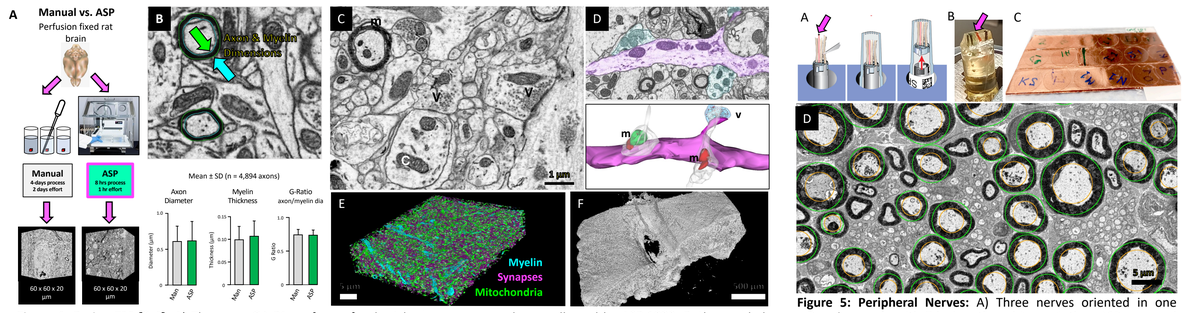

Figure 4: Brain vEM [3-4]: A) The same GA-PA perfusion-fixed rat brain was prepared manually and by ASP-1000. Both provided high-quality staining, preservation, infiltration, sectioning, and imaging of myelin, synaptic vesicles, and mitochondrial cristae. B) Axon and myelin dimensions were statistically 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 identification & quantification enabled with robotically uniform preparation. F) Correlative X-ray microscopy provides image of much larger volume.

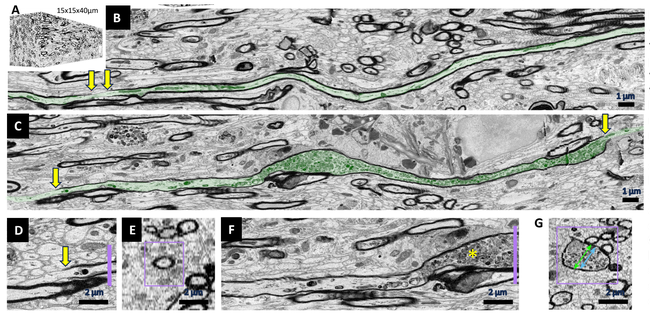

Figure 6: Axon and myelin nerve analysis in mouse corpus callosum after toxic demyelination: A) vEM stack. B) Intact axon (green) followed for ~40 µm by digitally reslicing vEM block. Node of Ranvier with arrows indicating paranodes. C) Axon (green) with isolated myelin internode (arrows= heminodes). D-G) Paranodal structures at higher magnification. D-E) Axon resliced transversely to measure axon diameter and thickness. Box in E shows plane of magenta line in D. F) Axon containing membranous debris (yellow asterisk). G) Reslicing transversely along magenta line shows distended axon with ~2.5 µm diameter (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). In isolation, this high G-ratio might suggest remyelination, but vEM analysis indicates pathologically swollen axon accumulating disrupted mitochondria and other organelles typical of impaired axonal transport.

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 B) resin embedded in the capsule (arrow). C) 1 µm thick sections are mounted and UA and Pb stained on coverslips, then arrayed on copper tape. D) SEM images with auto-segmented axons (E) [2].

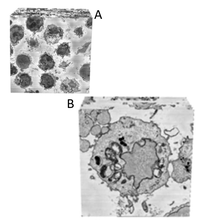

Figure 7: B-Cell Pellets: Splenic mouse B-Cells were pelleted, enrobed in low melting agarose, and ASP processed for vEM. A) Volume image of pellet. B) Individual B-cell [2, 8].

Automated Artificial Intelligence Analyses

The Core has developed neuro-specific and general structural biology specimen preparation and automated AI image analyses to accelerate discovery and assess experimental therapeutics.

Peripheral Nerves

Therapeutic trials typically require imaging 24 to 62 nerves. Sample prep is expedited by embedding up to 3 peripheral nerve segments (different experimental conditions) in a single mPrep/s capsule with a fiduciary thread (Fig. 5AB). These are then prepared using a ~4-hr TEM-like ASP protocol with 90 minutes OsO_4 for heavy myelin staining (Figure 5), and epoxy embedding (Fig. 5B). 1 µm sections are mounted on coverslips, stained with methanolic uranyl acetate and lead citrate, and arrayed on copper tape (Fig. 5C) for automated low kV backscattered SEM imaging that takes 24-48 hrs. Axon diameters, myelin thickness, and integrity/pathology using 2D section scanning are auto-segmented for therapy evaluations (Fig 5DE). Scripted imaging (Gatan Digital Micrograph) enables 30+ samples to be imaged at 20 nm/pixel in 1 hour. This provides automated quantitative axon pathology from multiple experimental conditions to assess therapeutics.

Internodal distances and myelin recovery in the corpus callosum

Toxic demyelination is a model used to test therapeutics that may impede axonal loss and increase remyelination (Fig. 6). Unlike peripheral nerves, where teased fiber preparations facilitate imaging of individual axons along their length, in the CNS, axons can not be easily separated. This mouse model enables 3D measures of internodal myelination of large-diameter motor axons by using vEM stacks, where individual axons are followed through multiple planes to measure myelin, detect myelin loss and repair, and document axonal damage. Figure 6B provides an example where a single axon is followed for ~40 µm, with much longer distances possible. A standard analysis would normally include 50 axons in 3 or more animals, to provide valuable metrics of therapeutic treatments.

B-Cells, Cell Pellets and Organoid vEM

Splenic B-Cells were pelleted, enrobed in low melting agarose, and ASP processed for vEM in ~8 hrs for vEM imaging (Fig. 6). This enrobing approach is also used for ASP-prep of neural organoids and cell pellets.

Summary

With ASP processing, the 3DEM core now employs a 3-step automated workflow comprising specimen prep, imaging, and concluding with deep-learning AI analysis. With this automated workflow, Core scientists now can focus on human-only knowledge tasks.

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