Automated Specimen Preparation for Volume Electron Microscopy

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Key Techniques: Automated specimen preparation for vEM. Correlative vEM, XRM and LM. Encapsulated specimen handling for automated & manual prep including freeze-substitution.

Keywords: Neuroscience, Cancer, Cell Biology, Planaria, Immunolabeling

Manual volume electron microscopy (vEM) specimen preparation using chemical fixation, staining, and embedding typically takes 4-5 days with one or more days of active “hands-on” effort that requires considerable handling of toxic reagents. This slows progress and takes time away from imaging, interpretation, analysis, and other more interesting work. Further, the tedium and complexity of manual preparation can lead to process inconsistencies which can negatively affect the reproducibility of results, including automated image segmentation.

We illustrate how several vEM labs use ASP-1000 and ASP-2000 Automated Specimen Processors and mPrep capsules to prepare biological specimens for serial block face-, array tomography- and FIB-SEM. Labs using ASPs report "hands-off" specimen prep as quick as 6 hours (prior to resin curing), with results equivalent or superior to manual prep, while providing the staining reproducibility needed for automated segmentation of mitochondria, myelin, synapses, and nuclei [1-4] with the safety of reduced reagent handling. ASP applications include automated prep of vertebrate and invertebrate tissues [1-4] and cell pellets [4-5]. Other labs use mPrep/s specimen capsules, without (or potentially with) ASP automation to improve freeze-substitution prep reliability by cutting loss and keeping specimens immersed [6]. We will also demonstrate how mPrep-encapsulated specimens can streamline correlative vEM with light and X-ray microscopy, and enable automated immunolabeling [7-8]. We gratefully acknowledge the contributions of ASP and mPrep capsule collaborators and customers

References:

1. Benson, E. *et al*. Serial Block-Face SEM of Brain Tissue Using Rapid Automated Preparation. *Microsc. Microanal*. **26(suppl 2)**, 1372-3 (2020).
2. Stempinski, E.S. *et al*. Automated large volume sample preparation for vEM. *Methods Cell Biology* **177**, 1-32 (2023).
3. McClain, M.L. & Nowotarski S.H. Serial block-face scanning electron microscopy of Schmidtea mediterranea. *Methods Cell Biology* **177**, 213-240 (2023).
4. Goodman, S.L, *et al.* Creating Efficient Workflows for Electron Microscopy Laboratories with Automated Specimen Preparation. *Microscopy Today,* **32:1**, 16-25 (2024).
5. Pal, A. *et al*. High Fat Diet-Induced Obesity Dysregulates Splenic B Cell Mitochondrial Activity. *Nutrients* **15**, 4807 (2023).
6. Bélanger, S. *et al.* A versatile enhanced freeze-substitution protocol for volume electron microscopy. *Front Cell Develop Bio*. **10**, 1-13 (2022).
7. Marques, P. *et al.* Optimization of Automated Immuno EM for Both Pre- and Post-Embedding Labeling. *Microsc Microanal* **24**, 1300-1 (2018).
8. Lillehoj, E.P. *et al*. Neuraminidase 1–mediated desialylation of the mucin 1 ectodomain releases a decoy receptor that protects against *Pseudomonas aeruginosa* lung infection. *J Biol Chem*. **294(2)**, 662-8 (2019).

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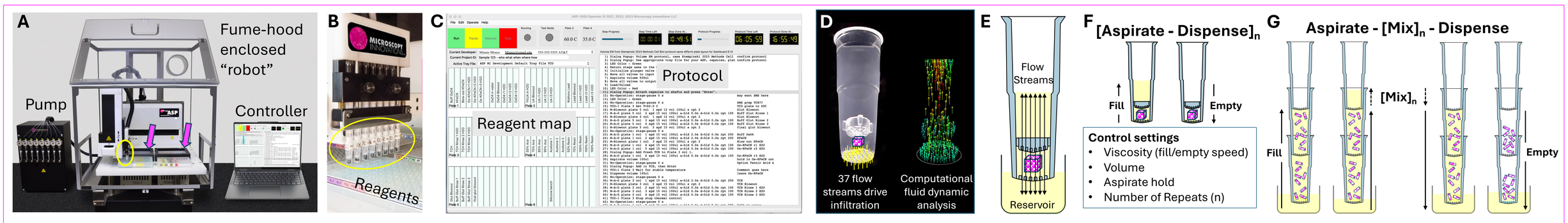
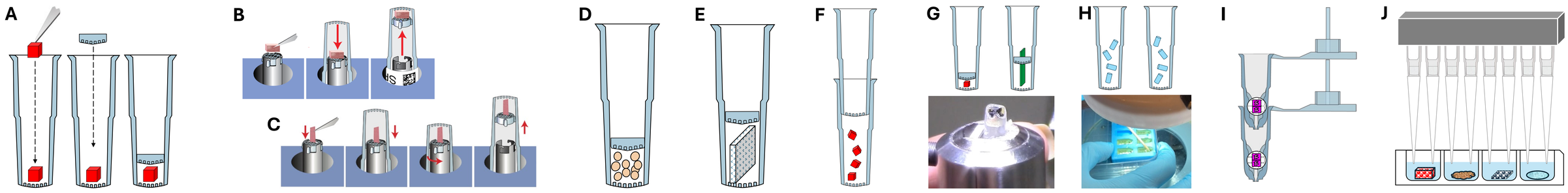


ASP™-1000 & ASP™-2000 Automated Specimen Processors. **A**) Purpose-built robot & pump deliver reagents to specimens in capsules on 8-channel head (circled) (**B**) from reagents in microplates. Arrows show two 0-100°C controlled reagent plates on ASP-2000. **C**) Intuitive user-modifiable & sharable protocols.). **D-E)** Bi-directional microfluidic flow for uniform rapid reagent infiltration. **F-G)** Mixing mode options: *Aspirate-Dispense* **(F)** for any number of cycles (control settings) provides very rapid prep for robust specimens. *Aspirate-Mix-Dispense* **(G)** for multiple specimens per capsule, several stacked capsules, and delicate specimens. ASPs prepare up to 128 TEM specimens in ~3 hrs, or 32 vEM specimens in ~6-8 hrs [1-6].



Purpose-built Specimen and Grid Capsules for most Workflows. A) mPrep/s (specimen) capsules entrap tissue, agar-enrobed specimens, pellets, organoids, etc. with mPrep/s screens placed with an insertion tool (not shown) or using Workstation **(B-C)**. Workstation optionally orient s specimens by **B**) entrapping between capsule bottom and screen, or **C**) by pinching the back of long specimens, e.g. nerve fibers, cell culture substrates). Or: **D**) Entrap several specimens. **E**) Planar specimens, such as cells on coverslips. **F**) High-throughput prep with 2 to 8 specimens in each capsule capped with a second capsule. Also used to trap high-pressure frozen specimens/planchets for cryo freeze-substitution [9]. **G)** Specimens entrapped/oriented with screens for embedding & sectioning in capsule. **H)** Remove specimens for flat embedding. **I**) TEM grids in mPrep/g capsules, such as for immunogold labeling [10-11]. **J**) Specimens larger than ~4 x 9 mm, e.g. large tissue specimens and whole coverslips are processed in 24-well plates using pipetting reagent delivery & agitation [12].

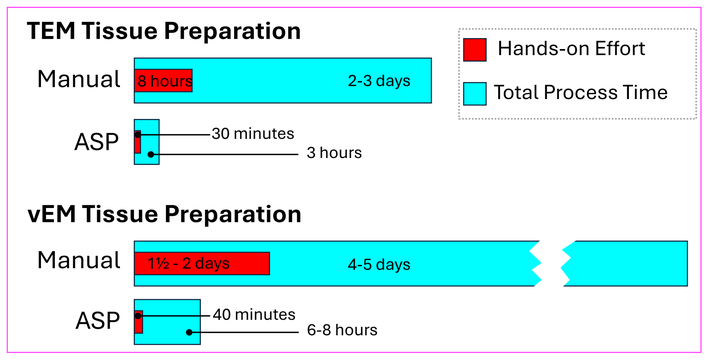
Problem

Manual chemical prep for volume electron microscopy (vEM) takes ~4-5 days with ~2 days of active "hands-on" effort. This slows progress and takes time away from imaging, analysis, publishing, and project management. The tedium and complexity of manual prep can also lead to process inconsistencies which can negatively affect reproducibility and image segmentation.

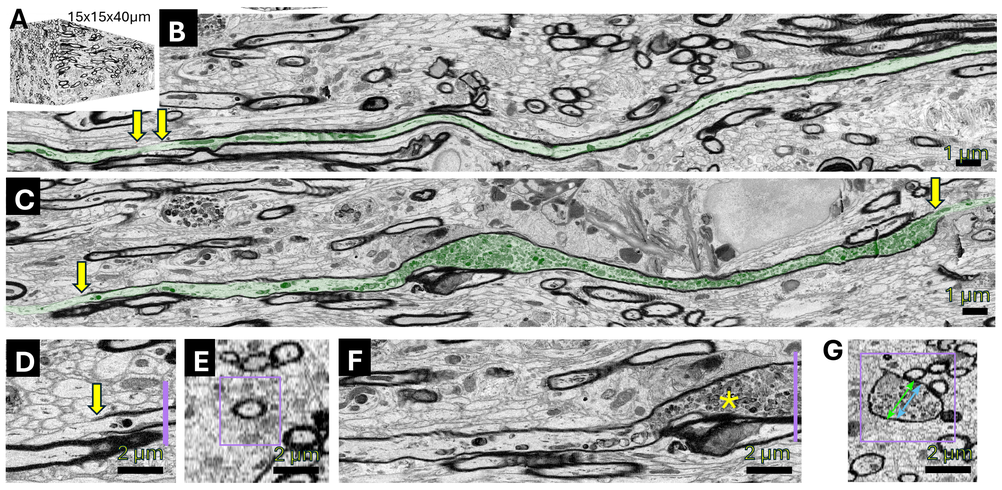
Solution

We show how several labs use mPrep™ ASP™-1000 & ASP™-2000 Automated Specimen Processors for vEM preparation. Labs using ASPs report "hands-off" vEM specimen prep as quick as 6 hours (before resin curing), with results equivalent or superior to manual prep, with staining reproducibility for automated segmentation and the safety of reduced reagent handling [1-4]. Examples shown include ASP prep of vertebrate and invertebrate tissues [1-7] and cell pellets [6,8]. Not shown are how mPrep capsules improve reliable freeze-substitution [9], streamline correlative light and X-ray microscopy, automate immunolabeling [10-11], and prepare very large tissue specimens and whole coverslips [12].

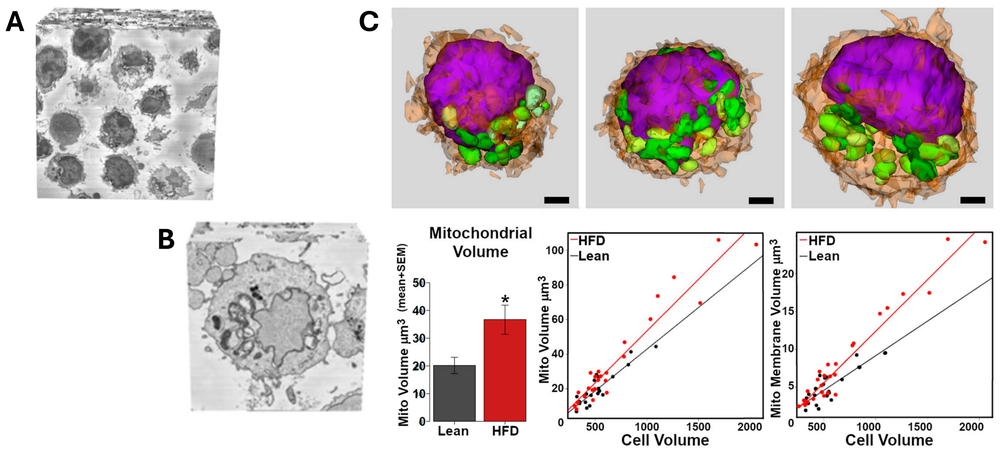
mPrep™ ASP™-1000 & ASP™-2000 processors are purpose-built to meet the demanding workflow needs of small and large EM labs, for nearly any specimen preparation task. ASPs are built for reactive EM reagents: OsO_4, RuO_4, TCH, solvents, and resins, unlike lab robots meant for aqueous biology applications. ASP durability has been proven over nearly a decade.



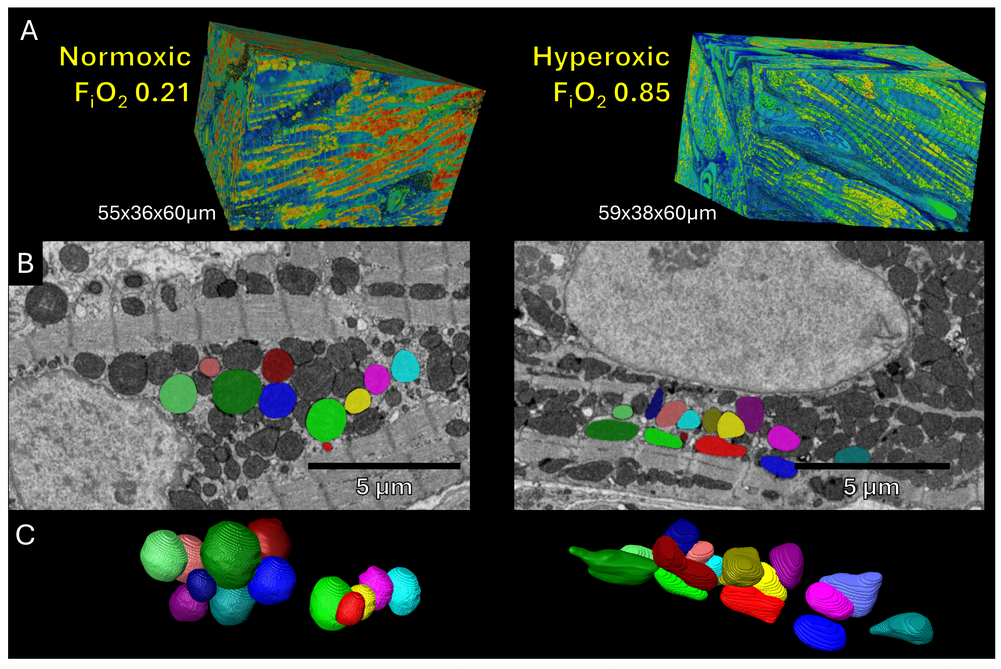
Specimen Prep Time & Effort: Typical process times and cumulative hands-on effort for manual & ASP prep for TEM & vEM, from primary aldehyde fixative rinse-out to 100% resin infiltration.



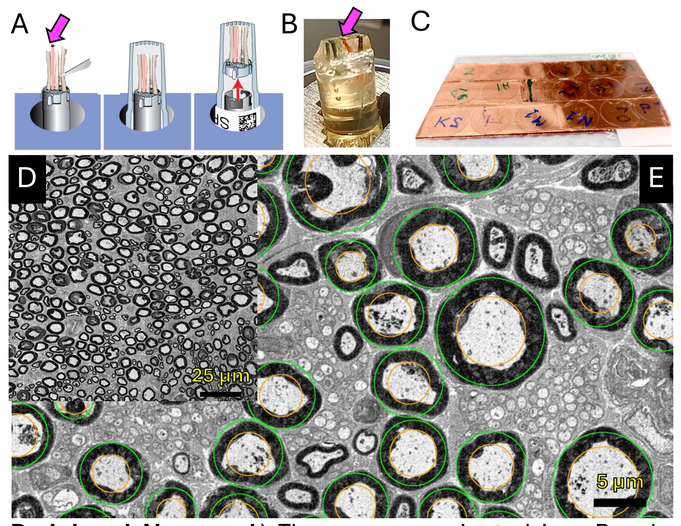
Axon & myelin nerve analysis after toxic demyelination. A) vEM stack from mouse corpus callosum. B) Intact axon (green) followed for ~40 µm by digitally reslicing vEM block. Arrows: Node of Ranvier paranodes. C) Axon (green) with isolated myelin internode (arrows=heminodes). D-G) Paranodal structures 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). 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. Benson E & Kidd G. 3DEM Ultrastructural Imaging Core, Cleveland Clinic, unpublished.



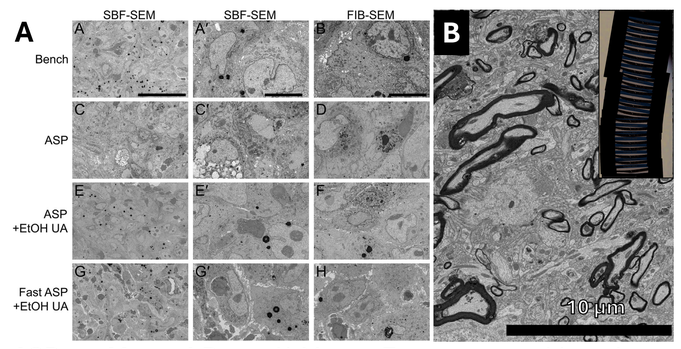
B-Cell Pellets: Splenic mouse B-Cells were pelleted, enrobed in low melting agarose, and ASP-1000 processed for serial block face vEM. A) Serial block face volume image of pellet. B) Individual B-cell, C) Segmentation and analysis. Mitochondria = green, Nucleus = Purple. 3DEM Ultrastructural Core, Cleveland Clinic [6,8].



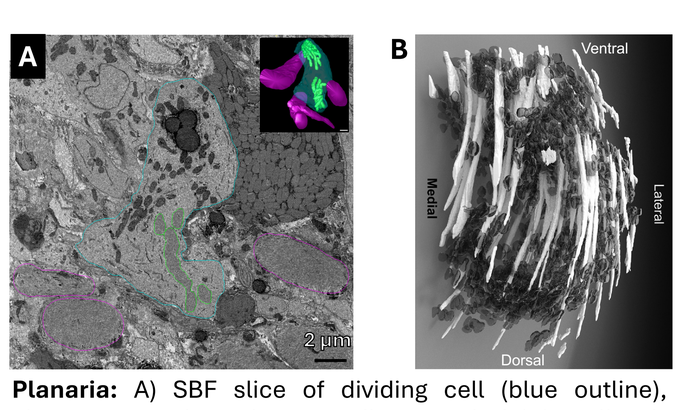
Heart right ventricle from neonate ICU model: vEM from control 'normoxic' FiO_2 neonate and 'hyperoxic' ICU rat pups. A) vEM 25 μm^3 cubes. B) Single slice vEMs with segmented mitochondria. C) 3D-rendered mitochondrial shapes differ between normoxic & hyperoxic. ASP prep 7.5 hr before resin curing from perfusion-fixed rats [5]. U. Wisconsin, Microscopy Innovations, ThermoFisher.



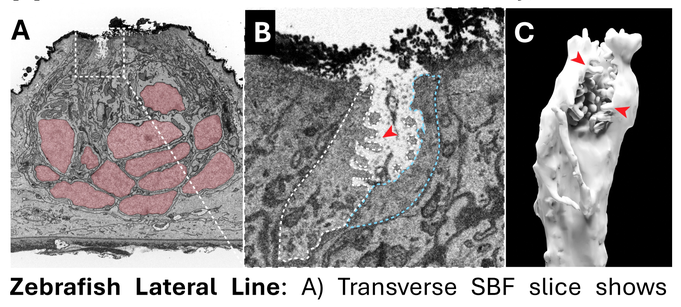
Peripheral Nerves: A) Three nerves oriented in mPrep/s capsule with fiduciary thread (arrow), then ASP-prepared for vEM including B) resin embedding in capsule (arrow). C) 1 µm sections mounted & UrAc-Pb stained on coverslips & arrayed on copper tape. D-E) BS-SEM images & auto-segmented axons for G-Ratio measurement [1,6].



ASP vs. Manual Prep: A) Mouse Tumor, manual bench vs. 3 ASP protocols, SBF & FIB-SEM imaging. B) Marmoset brain array tomography (inset=sections). "The ASP-2000…delivers on its promise to decrease the time required for specimen preparation for vEM, without compromising the quality of the specimens and images acquired [2]. Multiscale Microscopy Core, Oregon Health & Science University, USA.



Planaria: A) SBF slice of dividing cell (blue outline), chromosomes (green) surrounding muscle (pink outlines). Inset = 3D model [4]. B) Muscle fibers (white), nuclei (grey) [3]. Stowers Institute Medical Res. Kansas City, MO, USA.



Zebrafish Lateral Line: A) Transverse SBF slice shows apical part of a pair of neuromast ionocytes (white square) and cells (red nuclei). B) High mag (white square) shows microvilli (red arrowhead) and apical crypt of ionocyte pair exposed to neuromast outside. C) 3D microvilli model (red arrowheads) containing Notch cell crypt [7]. Stowers Inst. Pontificia U. Ecuador, U. Massachusetts, Virginia Tech.

References

1. Benson E *et al*. *Microsc. Microanal*. **26(2)**, 1372-3 (2020).
2. Stempinski ES *et al*. *Methods Cell Bio* **177**, 1-32 (2023).
3. McClain ML & Nowotarski SH. *Methods Cell Biol* **177**, 213-240 (2023).
4. McClain, ML *et al*. *Microsc. Microanal,* PDP-52 (2019).
5. Goodman SL *et al*. *Microsc. Microanal,* PDP-46 (2019).
6. Goodman SL *et al*. *Micro Today,* **32:1**, 16-25 (2024).
7. Peloggia J et ak. Develop Cell, 56(9) 1296-1312 (2021).
8. Pal A. *et al*. *Nutrients* **15**, 4807 (2023).
9. Bélanger S *et al*. *Front Cell Develop Bio*. **10**, 1-13 (2022).
10. Marques P. *et al*.. *Microsc Microanal* **24**, 1300-1 (2018).
11. Lillehoj EP *et al*. *J Biol Chem*. **294(2)**, 662-8 (2019).
12. Bleher R. Tech Talk: Nuance Ctr, Northwestern U. (2024).