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Automatic Sample Processing for vEM in a Mouse Model of Breast Cancer

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Volume electron microscopy (vEM) for biological samples requires intensive staining protocols to ensure both good contrast and conductivity throughout the entire sample. We have previously shown that the large volume en-bloc staining protocol developed by Hua et al. [1] has been used successfully for human cancer biopsies for both serial block face-scanning electron microscopy (SBF-SEM) and focused ion beam-scanning electron microscopy (FIB-SEM) [2]. However, this protocol requires 2.5 days of bench processing, which can be onerous in a microscopy core facility that may have multiple projects to complete. Additionally, we have previously compared the effectiveness of different bench protocols for vEM on breast cancer tumors [3] and observed that shorter processing time results in inadequate sample contrast and conductivity for SBF-SEM (unpublished data).

In our investigations we observed that the mPrep™ ASP-1000™ automated specimen processor from Microscopy Innovations can reduce the sample preparation time for vEM to roughly 24 hours [4]. We attempt to adapt the bench protocol used in our laboratory for the ASP-1000 and further refine the protocol to use shorter staining times and ethanolic uranyl acetate (UA) as in Thomas et al. [5] to understand morphological changes in a murine breast cancer model [6].

In general, we have observed that ASP-1000 vEM sample automation methods can produce similar results to the bench process in less time, with ethanolic UA increasing membrane contrast. Protocols performed on the ASP-1000 took 4.5 to 5 hours, with an hour of instrument setup and an hour of embedding and cleanup, for a total time of 7 hours of sample preparation time. In contrast, the bench protocol required 20 hours, not including time spent on the overnight steps. Active technician time for the ASP-1000 was 2.5 hours, whereas the bench protocol required over 5.5 hours of technician time. Additional advantages of the ASP-1000 include the reduced exposure of the operator to heavy metals and reproducibility of the automated process.

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[5] Thomas et al., Microscopy and Microanalysis **27** (2021), p. 156. doi: 10.1017/S1431927620024757
[6] Federico et al., Science Advances [Online] **3** e1600957 (2017), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5397135/ (accessed February 11, 2022).
[7] Electron microscopy was performed at the Multiscale Microscopy Core, a member of the OHSU University Shared Resource Cores. Funding was graciously provided by the OHSU Center for Spatial Systems and the NCI Cancer Systems Biology Measuring, Modeling, and Controlling Heterogeneity (M2CH) Center awarded to Joe Gray (5U54CA2099880) and NIH-NCI Cancer Center Support Grant (CCSG) 5P30CA069533-20. Special thanks to Dong Zhang in the OHSU Mills Lab for providing tissue.

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Abstract

- Volume EM (vEM) requires intensive staining protocols for contrast and conductivity.
- The Hua method (Hua, Laserstein & Helmsteder, 2015) can be used for both serial block face (SBF-) and focus ion beam scanning electron microscopy (FIB-SEM) but requires 2.5 days of bench processing before polymerization.
- Ethanolic uranyl acetate (UA) has been shown to increase membrane contrast (Thomas *et al*, 2021).
- Microscopy Innovations LLC mPrep™ ASP-1000™ automated specimen processor can reduce sample preparation time.

Methods

- Pieces of breast cancer tumor from a mouse model were fixed in 2.5% glutaraldehyde and 2.5% formaldehyde in 0.1M sodium cacodylate buffer and processed according to either the Hua bench protocol or in the mPrep ASP-1000 (shown in Figure 1) as outlined in Table 1.
- Samples were coated with 8 nm carbon and imaged on a Thermo Fisher Scientific Helios NanoLab G5 DualBeam FIB-SEM using a CBS detector at 3 kv, 0.2 nA probe current, 4 mm working distance, and a dwell time of 3 µs.

Table 1. A brief description of each major step of the Hua bench and mPrep ASP-1000 protocols, including time and temperature. RT denotes room temperature.

	Hua Bench Protocol			mPrep ASP-1000 Automatic Protocol		
Step	**Description**	**Temp (°C)**	**Time**	**Description**	**Temp (°C)**	**Time**
Reduced osmium tetroxide	2% OsO_4 in buffer	RT	1.5 hr	2% OsO_4 in buffer	RT	0.25 hr
	2.5% potassium ferricyanide in buffer	RT	1.5 hr	2.5% potassium ferricyanide in buffer	RT	0.25 hr
Thiocarbohydrazide	1% TCH	40°C	0.75 hr	1% TCH	60°C	0.25 hr
Osmium tetroxide	2% OsO_4 in water	RT	1.5 hr	2% OsO_4 in water	RT	0.25 hr
Uranyl acetate	1% UA aqueous	4°C, 50°C	Overnight + 2 hr	1% UA aqueous or 1% UA in 25% ethanol	RT	0.25 hr
Walton's lead stain	---	50°C	2 hr	---	60°C	0.25 hr
Dehydration	Acetone: 50%, 75%, 95%, 100%	RT	1 hr	Acetone: 50%, 75%, 95%, 100%	RT	0.25 hr
Infiltration	Acetone:Resin 1:1, 1:3, Pure resin x4	RT	3.3 hr + overnight	Acetone:Resin 1:1, 1:3 Pure resin x3	RT	2.25 hr
Total time before polymerization, excluding overnight time:			15.9 hr			5 hr

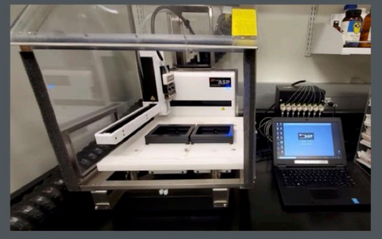

Figure 2. The Microscopy Innovations mPrep ASP-1000 automated sample processor with a thermal control unit.

Figure 3. Reagent tray and specimen capsule for the mPrep ASP-1000

Results and Discussion

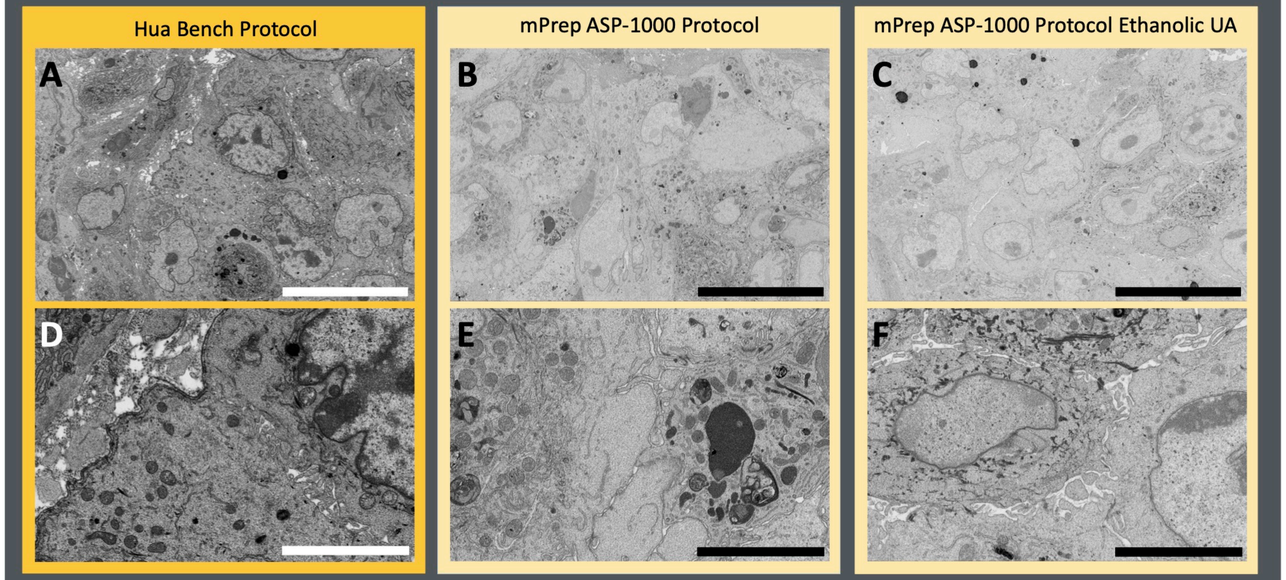

Figure 4. Contrast comparison between the Hua bench protocol (A, D), the ASP-1000 protocol (B, E) and the mPrep ASP-1000 protocol with ethanolic UA (C, F). Samples were imaged concurrently with the same brightness and contrast. Scale bar = 20 µm for A, B, C and scale bar = 5 µm for D, E, F.

- The mPrep ASP-1000 automated sample processor reduces both technician time and overall sample processing time for vEM protocols to three hours of technician time and five hours of processing time.
- Ethanolic UA allows for greater contrast of membranes, possibly at the expense of cytosolic staining.
- The mPrep ASP-1000 allows for adequate contrast for FIB-SEM. If needed, greater signal and contrast can be achieved by incubating samples with stain for longer. This five-hour automated protocol is nearly equivalent to bench processing for thin brain slices (data not shown).

Acknowledgements

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