Automated Heavy Metal Tissue Staining for Serial Block Face Imaging with the ASP-1000

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Electron microscopy techniques for obtaining three-dimensional information are providing new insights into the ultrastructure of many tissue types. Many of these techniques involve long incubations in heavy metals before embedding in resin. Here we show one way to automate these long staining and processing protocols by using the ASP-1000 sample processing robot developed by Microscopy Innovations. Using the ASP-1000 with a plate position heating feature, we show it's potential to effectively stain samples for serial block face imaging of the flatworm *S. mediterranea*. With alterations for different sample types and different staining needs, the ASP-1000 could free up specialist time and provide greater consistency between samples and runs, as well as faster turnaround time.

The ASP-1000 robot setup includes preparing plates with the reagents and placing in the appropriate positions for the program to incubate the samples in the proper order, and loading mPrep/s capsules with samples onto the pipetting arm (Figure 1). Incubations with acetone or ethanol are covered with pierceable aluminum plate covers using a $4s^3$ plate sealer from 4titude® Ltd., and heated steps are covered with 4titude X-Pierce sealing films to prevent evaporation. Staining schedules were inspired by published protocols and adapted to increase staining [2,3]. Constant agitation was used on all steps. Samples were either embedded directly onto a sample pin with 2 part silver conductive epoxy and cured at 60°C for 48 hours, or flat embedded at 60°C for 48 hours and mounted on sample pins with the silver epoxy after curing. Sample pins were faced and trimmed with a diamond trim tool from Diatome and serial block face imaging was performed on a Zeiss Merlin with the Gatan 3View 2XP system at 2.5 kV in high resolution mode with an I Probe setting of 167 pA. Images were taken at 10 nm pixel size with a section thickness of 50 nm and pixel dwell time of 2 us, using the Zeiss Focal Charge Compensator at 30% nitrogen gas to reduce sample charging. Stacks were aligned and modeled with IMOD [2].

Image stacks from samples processed with the ASP-1000 have performed similarly to bench processed samples, with good contrast and cutting properties. Ultrastructure preservation is good, and cellular components are visible for 3D modelling (Fig. 2). By moving the sample processing protocol to an automated platform, we have shortened the overall processing time by two days. We have traded almost five days of specialist time manually pipetting at the bench for one half day of plate preparation and sample loading, enabling specialists to concentrate on other tasks while the robot works at all hours of the day. Creating robot programs of staining protocols appropriate for other sample types and purposes is currently under way.

References:

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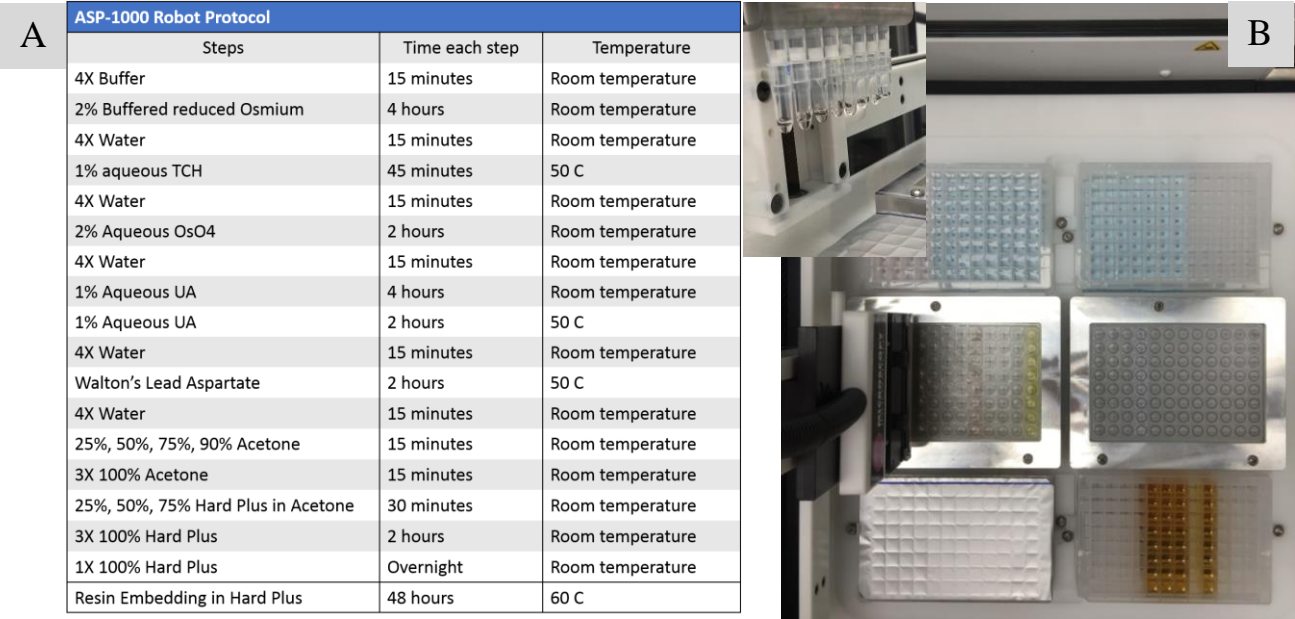
Figure 1. (A) Processing steps and (B) set up for *S. mediterranea* samples processed on the ASP1000. Tray positions for 100% resin steps were left uncovered and filled while the program was running, close to the time for those steps.
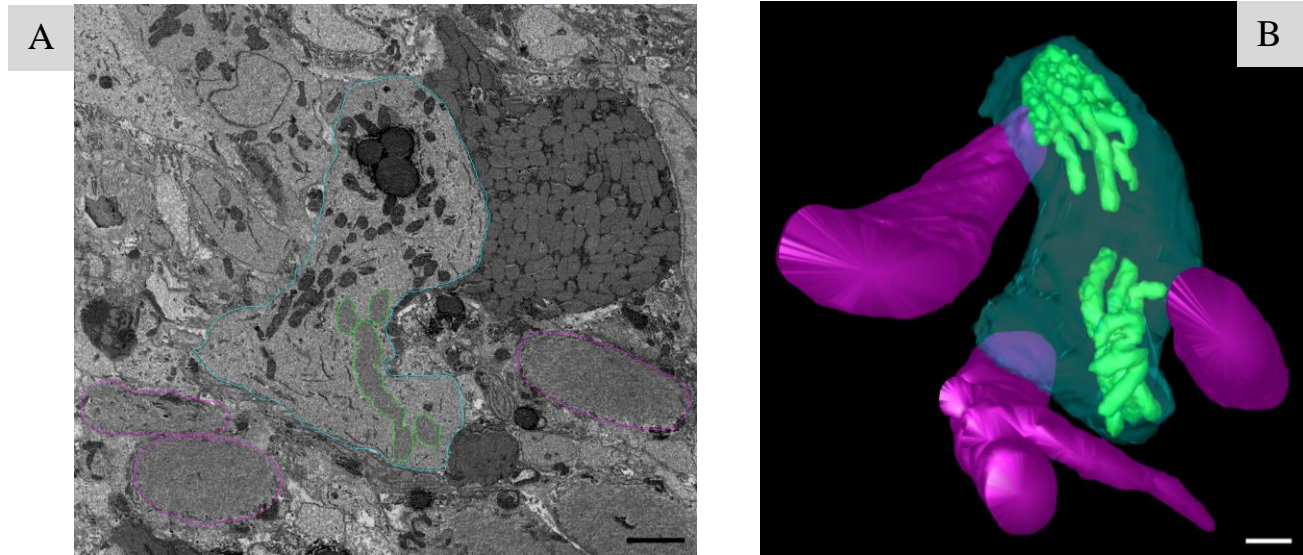

Figure 2. (A) Image of a slice from a serial block face volume processed on the ASP1000 robot showing a dividing cell outlined in blue, chromosomes outlined in green, and surrounding muscles outlined in pink. (B) 3D mesh showing the model created by tracing from the image volume. Scale bar in both images is 2 um.

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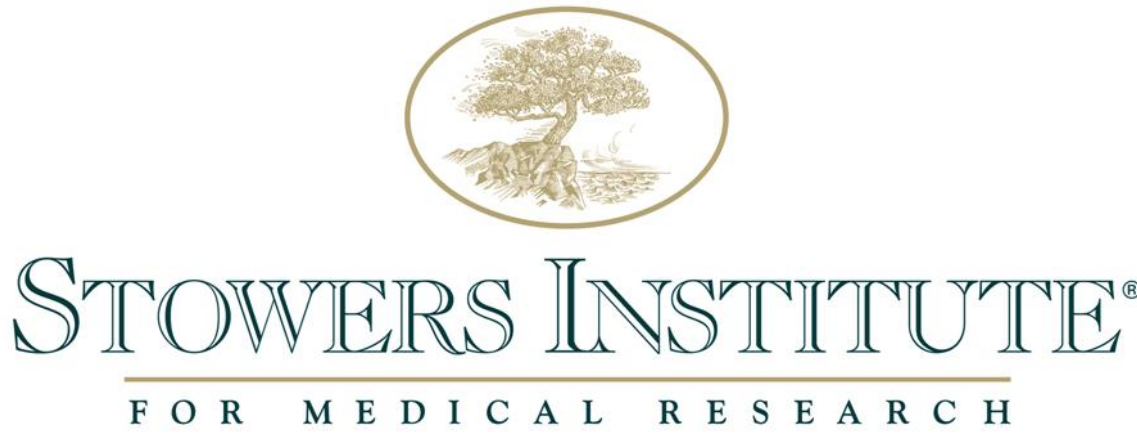
Introduction

Electron microscopy techniques for obtaining three-dimensional information are providing new insights into the ultrastructure of many tissue types. Many of these techniques involve long incubations in heavy metals before embedding in resin. Here we show one way to automate these long staining and processing protocols by using the ASP-1000 sample processing robot developed by Microscopy Innovations. Using the ASP-1000 with a plate position heating feature, we show it's potential to effectively stain samples for serial block face imaging of the flatworm *S. mediterranea*. With alterations for different sample types and different staining needs, the ASP-1000 could free up specialist time and provide greater consistency between samples and runs, as well as faster turnaround time.

Experiment

The ASP-1000 robot setup includes preparing plates with the reagents and placing in the appropriate positions for the program to incubate the samples in the proper order, and loading mPrep/s capsules with samples onto the pipetting arm (Figure 1). Incubations with acetone or ethanol are covered with pierceable aluminum plate covers using a 4s³ plate sealer from 4titude® Ltd., and heated steps are covered with 4titude X-Pierce sealing films to prevent evaporation. Staining schedules were inspired by published protocols and adapted to optimize both membrane and chromosome staining [2,3]. Constant agitation was used on all steps. Samples were either embedded directly onto a sample pin with 2 part silver conductive epoxy and cured at 60°C for 48 hours, or flat embedded at 60°C for 48 hours and mounted on sample pins with the silver epoxy after curing. Sample pins were faced and trimmed with a diamond trim tool from Diatome and serial block face imaging was performed on a Zeiss Merlin with the Gatan 3View 2XP system at 2.5 kV in high resolution mode with an I Probe setting of 167 pA. Images were taken at 10 nm pixel size with a section thickness of 50 nm and pixel dwell time of 2 us, using the Zeiss Focal Charge Compensator at 30% nitrogen gas to reduce sample charging. Stacks were aligned and modeled with IMOD [2].

ASP-1000 Robot Protocol

| Steps	Time each step	Temperature
4X Buffer	15 minutes	Room temperature
2% Buffered reduced Osmium	4 hours	Room temperature
4X Water	15 minutes	Room temperature
1% aqueous TCH	45 minutes	50 C
4X Water	15 minutes	Room temperature
2% Aqueous OsO4	2 hours	Room temperature
4X Water	15 minutes	Room temperature
1% Aqueous UA	4 hours	Room temperature
1% Aqueous UA	2 hours	50 C
4X Water	15 minutes	Room temperature
Walton's Lead Aspartate	2 hours	50 C
4X Water	15 minutes	Room temperature
25%, 50%, 75%, 90% Acetone	15 minutes	Room temperature
3X 100% Acetone	15 minutes	Room temperature
25%, 50%, 75% Hard Plus in Acetone	30 minutes	Room temperature
3X 100% Hard Plus	2 hours	Room temperature
1X 100% Hard Plus	Overnight	Room temperature
Resin Embedding in Hard Plus	48 hours	60 C
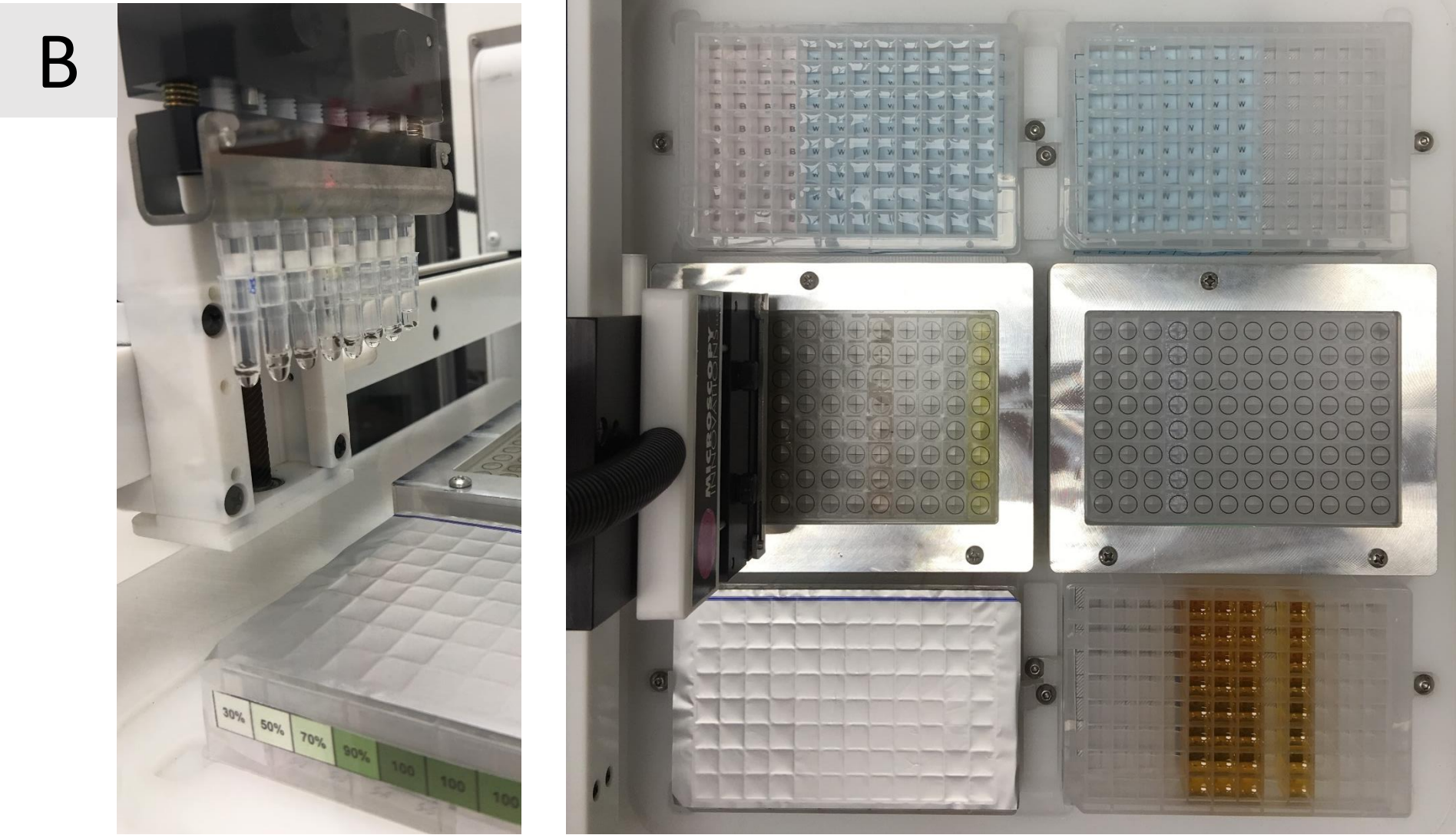
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[6] The authors acknowledge funding from the Stowers Institute for Medical Research, and HHMI.

Conclusion

Image stacks from samples processed with the ASP-1000 have performed similarly to bench processed samples (Fig. 3), with good contrast and cutting properties. Ultrastructure preservation is good, and cellular components are visible for 3D modelling (Fig. 2). By moving the sample processing protocol to an automated platform, we have shortened the overall processing time by two days. We have traded almost five days of specialist time manually pipetting at the bench for one half day of plate preparation and sample loading, enabling specialists to concentrate on other tasks while the robot works at all hours of the day.
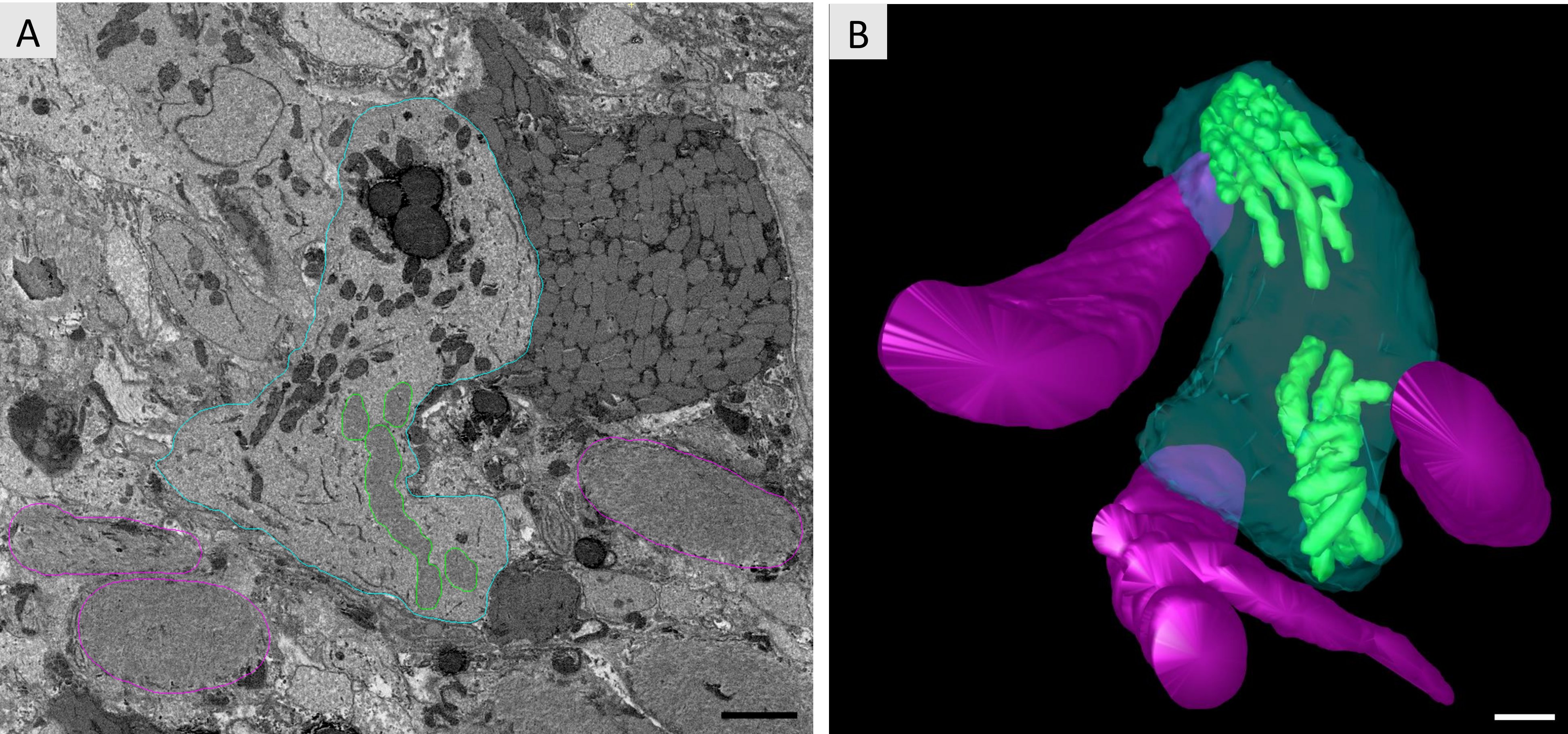

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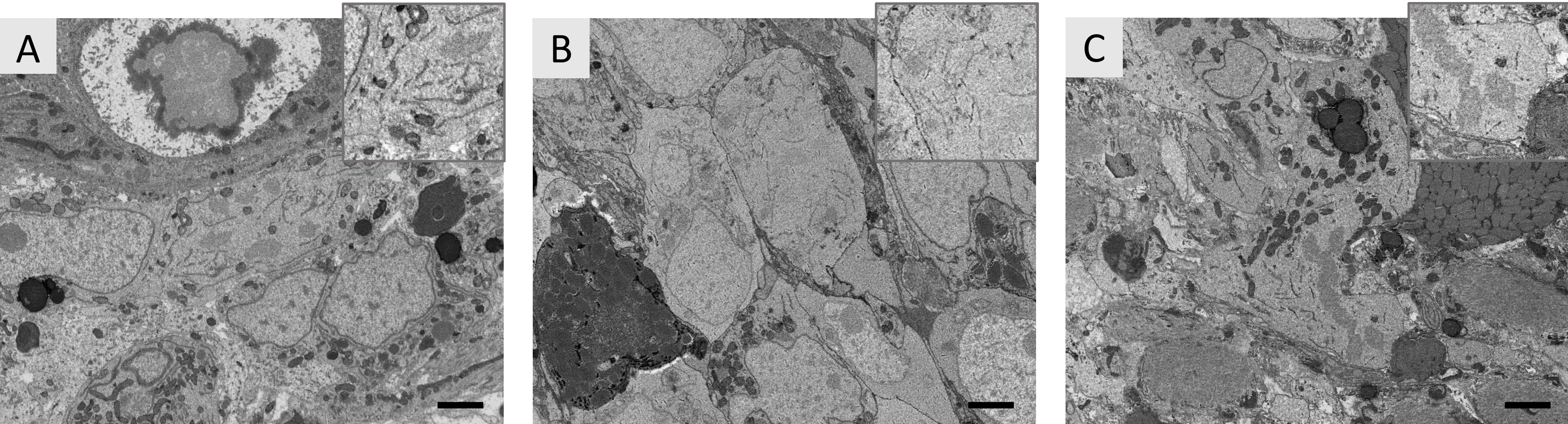

Figure 3. Images (A) and (B) from serial block face volumes processed on the bench using protocol [2] for (A) and [3] with extended staining times for (B). Image (C) from ASP-1000 robot protocol shows equivalent staining and ultrastructure preservation. Inserts show close-ups of the staining in each image. Scale bar is 2 um.