Rapid Automated en Bloc Staining for SEM of Sections

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Increased interest in 3D reconstruction of biological tissues has encouraged many specialized *en bloc* staining protocols for increasing contrast and conductivity of tissue blocks, allowing collection of large 2D-3D datasets at high resolution by SEM [1]. Current *en bloc* staining methods for 3D reconstruction of biological samples use multiple steps requiring hands on time and reagent temperature changes. Here we describe a protocol for rapid, automated *en bloc* staining for plastic embedded tissue using the mPrep ASP-1000 robot. With automated rapid agitation, this protocol achieves similar *en bloc* staining in less time without temperature changes, while eliminating manual pipetting, freeing up specialist time.

Whole planarian flatworm animals, *Schmidtea mediterranea,* were processed using a modified automated protocol for rapid TEM sample preparation developed by the author, combined with the OTO and Walton's lead aspartate staining steps of the Ellisman protocol, a popular staining *en bloc* staining protocol [2]. Adult, asexual *Schmidtea mediterranea* between 1.5mm-2.5mm were rinsed once with Montjiuc water, fixed on a cold plate and loaded into mPrep capsules (Fig. 2). Well plates were loaded with reagents and samples were processed with rapid continuous agitation for each step (Fig. 1), facilitating shortened times compared to the bench protocol. After dehydration in acetone, samples were infiltrated with Epon resin (Electron Microscopy Sciences) and embedded the next morning at 100^{o}C for 3-4 hours. Sections were cut on a Leica UC6 ultramicrotome at 80 nm, mounted on slides, and coated with carbon, and were imaged on a Zeiss Merlin SEM in BSE mode at 4 kV and 440 pA with a working distance of 7.8 mm.

Figure 2 shows samples processed with the rapid robot protocol and manually with the Ellisman protocol. The automated protocol provided good preservation of cellular organelles such as mitochondria and ER, and regularity of membranes such as the nuclear envelope were comparable between protocols. Staining intensity was less than the longer bench protocol, but produced easy to section blocks which imaged well and were not brittle. The more work intensive bench protocol produced more contrast, but samples tended to be brittle making sectioning more difficult. For a different level of staining or staining of different cellular components, times and reagents can be easily changed.

Here we show a modified *en bloc* protocol at room temperature that produces sections of biological tissue samples suitable for imaging by SEM. The rapid sample processing time allows data collection from fixed samples in as little as 2 days. The automated nature of the ASP-1000 frees specialist time during sample processing, and the 8-channel capacity allows variation of reagents and concentrations across wells, facilitating fast optimization of protocols suitable for other species or applications such as serial block face methods and automated section collection technology.

References:

[1] Juan Carlos Tapia, *et al*, Nature Protocols **7** (2012), p. 193-206.
[2] Deerinck, T.J., *et al*, NCMIR methods for 3D EM: A new protocol for preparation of biological specimens for serial block face scanning electron microscopy, Microscopy, (2010), pg: 6-8

[3] The authors acknowledge funding from the Stowers Institute for Medical Research, and HHMI

A

ASP-1000 Robot Protocol			Manual Bench Protocol	
Time Each Step	Temperature	Steps	Temperature	Time Each Step
40 seconds	RT	4X Buffer	4 C	15 minutes
22 minutes	RT	2% Buffered OsO4	On Ice	1 hour
40 seconds	RT	3X Water	RT	5 minutes
minutes	RT	TCH	RT	20 minutes
40 seconds	RT	3X Water	RT	5 minutes
minutes	RT	2% Aqueous OsO4	RT	30 minutes
40 seconds	RT	3X Water	RT	5 minutes
6 minutes	RT	1% Aqueous UA	4 C	Overnight
40 seconds	RT	3X Water	RT	5 minutes
minutes	RT	Walton's Lead Aspartate	60 C	30 minutes
40 seconds	RT	3X Water	RT	5 minutes
40 seconds	RT	25%, 50%, 75% Acetone	On ice	10 minutes
40 seconds	RT	4X 100% Acetone	On ice	10 minutes
5 minutes	RT	25%, 50%, 75% Epon in Acetone	RT	1 hour to Overnight
10 minutes	RT	3X 100% Epon	RT	2 changes per day
Overnight	RT	1X 100% Epon	RT	2 changes per day
3 hours	100 C	Resin Embedding in Epon	60 C	48 hours
1.5 days				7 days

B

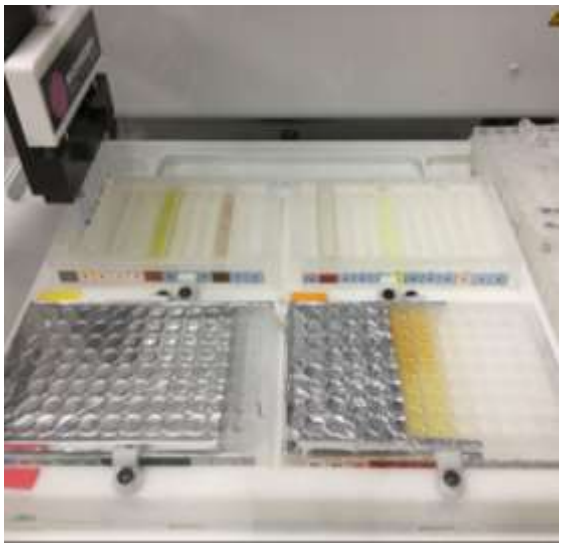

Figure 1. (A) Bench vs. automated times for each incubation step. The automated protocol makes it possible to image samples in as little as 2 days. (B) ASP-1000 robot set-up with reagents and samples loaded.

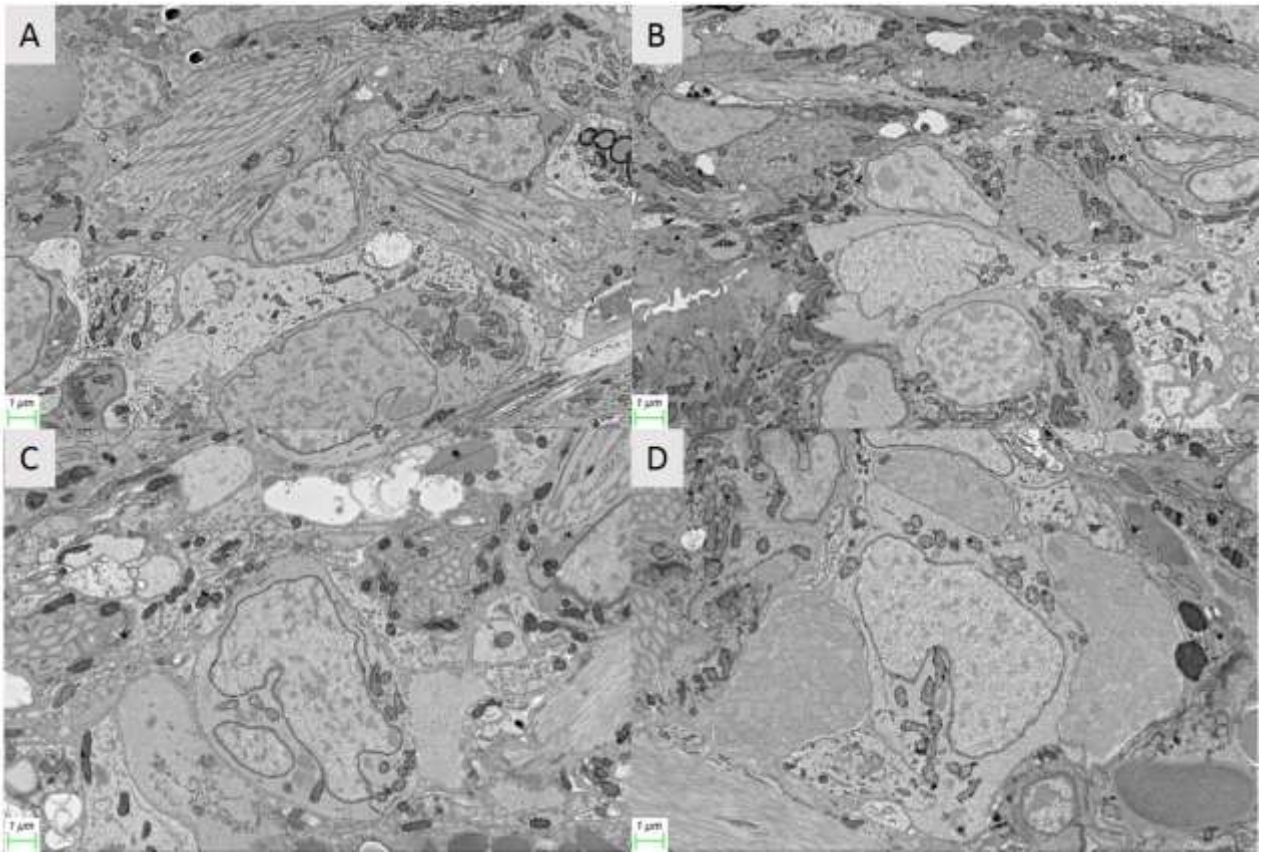

Figure 2. Bench protocol (A and C), and automated room temperature protocol (B and D). The automated protocol produces samples that are analogous to the bench protocol.

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Introduction

Increased interest in 3D reconstruction of biological tissues has encouraged many specialized *en bloc* staining protocols for increasing contrast and conductivity of tissue blocks, allowing collection of large 2D-3D datasets at high resolution by SEM [1]. Current *en bloc* staining methods for 3D reconstruction of biological samples use multiple steps requiring hands on time and reagent temperature changes. Here we describe a protocol for rapid, automated *en bloc* staining for plastic embedded tissue using the mPrep ASP-1000 robot. With automated rapid agitation, this protocol achieves similar *en bloc* staining in less time without temperature changes, while eliminating manual pipetting, freeing up specialist time.

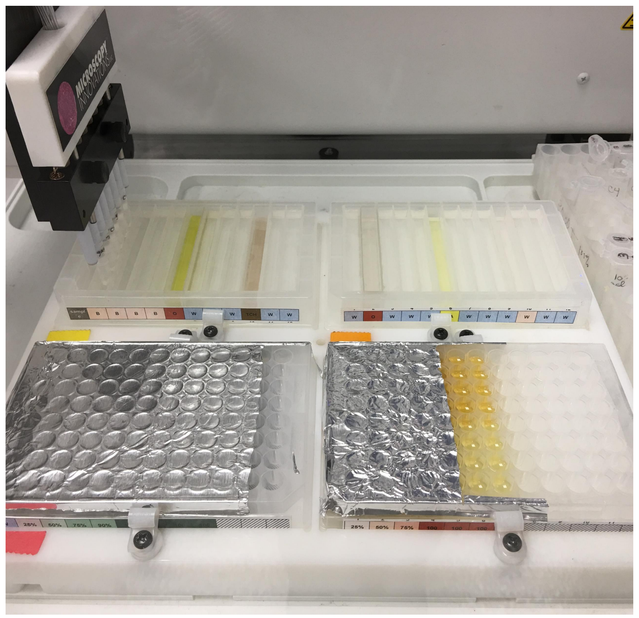

Figure 1. ASP-1000 robot set-up with reagents and samples loaded.

Experiment

Whole planarian flatworm animals, *Schmidtea mediterranea,* were processed using a modified automated protocol for rapid TEM sample preparation developed by the author, combined with the OTO and Walton's lead aspartate staining steps of the Ellisman protocol, a popular staining *en bloc* staining protocol [2]. Adult, asexual *Schmidtea mediterranea* between 1.5mm-2.5mm were rinsed once with Montjiuc water, fixed on a cold plate and loaded into mPrep capsules (Fig. 1). Well plates were loaded with reagents and samples were processed with rapid continuous agitation for each step, facilitating shortened times compared to the bench protocol (Fig. 3). After dehydration in acetone, samples were infiltrated with Epon resin (Electron Microscopy Sciences) and embedded the next morning at 100°C for 3-4 hours. Sections were cut on a Leica UC6 ultramicrotome at 80 nm, mounted on slides and coated with carbon, and were imaged on a Zeiss Merlin SEM in BSE mode at 4 kV and 440 pA with a working distance of 7.8 mm. Figure 2 shows samples processed with the rapid robot protocol and manually with the Ellisman protocol. The automated protocol provided good preservation of cellular organelles such as mitochondria and ER, and regularity of membranes such as the nuclear envelope were comparable between protocols. Staining intensity was less than the longer bench protocol, but produced easy to section blocks which imaged well and were not brittle. The more work intensive bench protocol produced more contrast, but samples tended to be brittle making sectioning more difficult. For a different level of staining or staining of different cellular components, times and reagents can be easily changed.

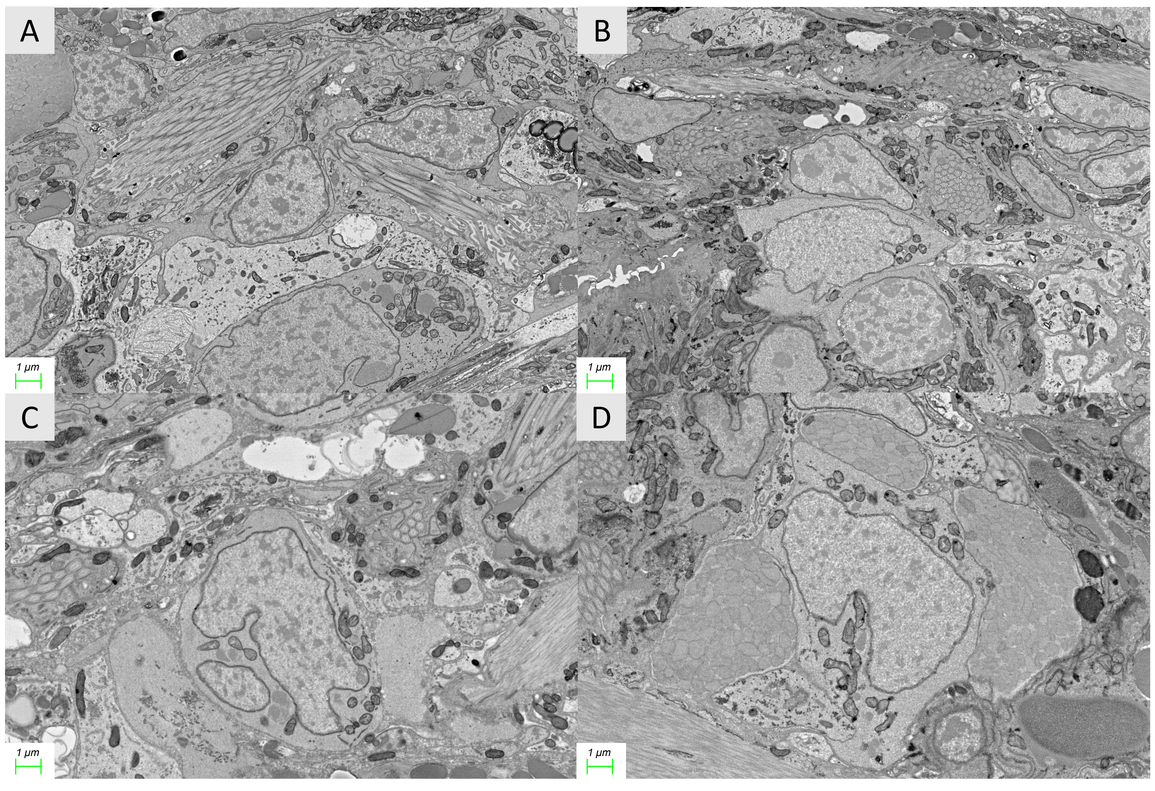

Figure 2. Bench protocol (A and C), and automated room temperature protocol (B and D). The automated protocol produces samples that are analogous to the bench protocol.

Conclusion

Here we show a modified *en bloc* protocol at room temperature that produces sections of biological tissue samples suitable for imaging by SEM. The rapid sample processing time allows data collection from fixed samples in as little as 2 days. The automated nature of the ASP-1000 frees specialist time during sample processing, and the 8-channel capacity allows variation of reagents and concentrations across wells, facilitating fast optimization of protocols suitable for other species or applications such as serial block face methods and automated section collection technology.

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