

Novel Pipette Based Capsule Processing Methods for Epoxy, Glycol Methacrylate, and Methyl/Butyl Methacrylate Resins with Mouse Tissue That Reduce Processing Volume and Time.

Philip Seifert HTL(ASCP), Xinyao Hu, Bianai Fan and Darlene A. Dartt, PhD, Schepens Eye Research Institute Morphology Core

Background

The mPrep/S capsules (Microscopy Innovations) are customized pipette tips with screen inserts designed for tissue sizes of ~1mm³ to fully process and embed in a fixed orientation for various electron microscopy applications (1). Chemical fixation and embedding is diffusion limited, by using capsules and pipette-propelled reagent exchanges, rapid processing can be achieved by accelerating diffusion of reagents/solutions into tissue specimens with repeated directed fluid flow (1). Capsule processing methods were developed for mouse ocular and mineralized bone for histology and transmission electron microscopy (TEM) applications and compared to conventional manual and carousel processed methods.

Methods

The novel methods tested enable processing larger sized tissues (~3mm diameter) including whole, anterior or posterior segments of mouse eyes within a single capsule that use 75% less reagents and time. Samples were fixed with; 1/2 Karnovsky's fixative (mouse ocular posterior segment, optic nerve[ON]), mod. Davidson's (mouse whole eye), or 10% NBF (mouse femur) for 48 hours then loaded into capsules using the mPrep/S workstation to screen pinch orient (ON) or secure unoriented. A programable electronic motor-assisted 12x 300uL- multi-channel pipette (VWR #10827-936) was utilized to process and embed into HR Technovit 7100 Glycol Methacrylate (GMA), methyl methacrylate/butyl methacrylate (MBMA, EMS #14520) and EMBED812 epoxy resin (#14120) using processing protocols listed in tables 1-4. Capsule processed tissue embedded in GMA, MBMA and Epoxy resins were sectioned for histological staining including H&E, toluidine blue, osmium-paraphenylenediamine (PPD), Von Kossa and TEM imaged. Ultrathin sections (80nm) were post-grid stained with uranyl acetate and Sato's lead stain (2) and imaged on a FEI Tecnai G2 Spirit TEM @80KV with a AMT XR41 digital camera. PPD stained semithin sections were imaged using a Leica SP8 confocal microscope and axons quantified using ImageJ-FIJI AxoNet software (3). Stained slide scanned micrographs (Hamamatsu Nanozoomer) and TEM images from capsule processed samples were compared to conventional processed using the EMS Lynx2 EM carousel processor or manual processing.

Table 1. Eye- Embed812 Epoxy				Table 2. Optic Nerve- Embed812 Epoxy				Table 3. Mouse Eye- GMA				Table 4. Mouse Femur- MBMA			
Step	Reagent	Fluid mix cycles	Time (min)	Step	Reagent	Fluid mix cycles	Time (min)	Step	Reagent	Fluid mix cycles	Time (min)	Step	Reagent	Fluid mix cycles	Time (min)
1	Fix Rinse 1 (Caco. Buffer)	100	3	1	Fix Rinse 1 (Caco. Buffer)	100	3	1	Rinse (DI water)	100	10	1	25% Ethanol	100	10
2	Fix Rinse 2 (Caco. Buffer)	100	3	2	Fix Rinse 2 (Caco. Buffer)	100	3	2	25% Ethanol	100	10	2	50% Ethanol	100	10
3	2% OsO4 in Buffer	600	90	3	2% OsO4 in Buffer	600	90	3	50% Ethanol	100	10	3	75% Ethanol	100	10
4	Rinse 1 (Caco. Buffer)	100	3	4	Rinse 1 (Caco. Buffer)	100	3	4	75% MBMA	100	10	4	95% Ethanol	100	10
5	Rinse 2 (DI water)	100	3	5	Rinse 2 (DI water)	100	3	5	95% Ethanol	100	10	5	95% Ethanol	100	10
6	2% Uranyl Acetate in DI Water	600	30	6	Rinse 3 (DI water)	100	3	6	95% Ethanol	100	10	6	100% Ethanol	100	10
7	Rinse 4 (DI water)	100	3	7	Rinse 4 (DI water)	100	3	7	100% Ethanol	100	10	7	100% Ethanol	100	10
8	25% Ethanol	100	3	8	25% Ethanol	100	3	8	100% Ethanol	100	10	8	100% Ethanol	100	10
9	50% Ethanol	100	3	9	50% Ethanol	100	3	9	25% GMA	200	15	9	25% MBMA	200	15
10	75% Ethanol	100	3	10	75% Ethanol	100	3	10	50% GMA	200	15	10	50% MBMA	200	15
11	95% Ethanol	100	3	11	95% Ethanol	100	3	11	75% GMA	200	15	11	75% MBMA	200	15
12	95% Ethanol	100	5	12	95% Ethanol	100	5	12	100% GMA	200	15	12	100% MBMA	400	15
13	100% Ethanol	100	5	13	100% Ethanol	100	5	13	100% GMA	400	15	13	100% MBMA	400	15
14	100% Ethanol	100	10	14	100% Ethanol	100	10	14	100% GMA Polymerize (External Mold)	24 hours		14	100% MBMA	400	15
15	100% Ethanol	100	3	15	100% Ethanol	100	3					15	100% MBMA	400	15
16	Propylene Oxide (PO)	100	3	16	Propylene Oxide (PO)	100	3					16	100% MBMA w/ catalyst solution	400	15
17	Propylene Oxide (PO)	100	3	17	Propylene Oxide (PO)	100	3					17	100% MBMA w/ catalyst solution (External mold) 65°C Oven Cure	24 hours	
18	25% Epoxy in PO	100	15	18	25% Epoxy in PO	100	15								
19	50% Epoxy in PO	100	15	19	50% Epoxy in PO	100	15								
20	75% Epoxy in PO	100	15	20	75% Epoxy in PO	100	15								
21	100% Epoxy	100	30	21	100% Epoxy	100	30								
22	100% Epoxy	100	30	22	100% Epoxy	100	30								
23	100% Epoxy (Capsule) 60°C Oven Cure	24 hours		23	100% Epoxy (Capsule) 60°C Oven Cure	24 hours									
Steps 1-22				Steps 1-22				Steps 1-13				Steps 1-16			
Time (hours)				Time (hours)				Time (hours)				Time (hours)			
Carousel Processing				Carousel Processing				Manual Processing				Manual Processing			
27.2				26.7				18.1				26.8			
Capsule Processing				Capsule Processing				Capsule Processing				Capsule Processing			
4.7				3.8				2.6				3.3			

Methods

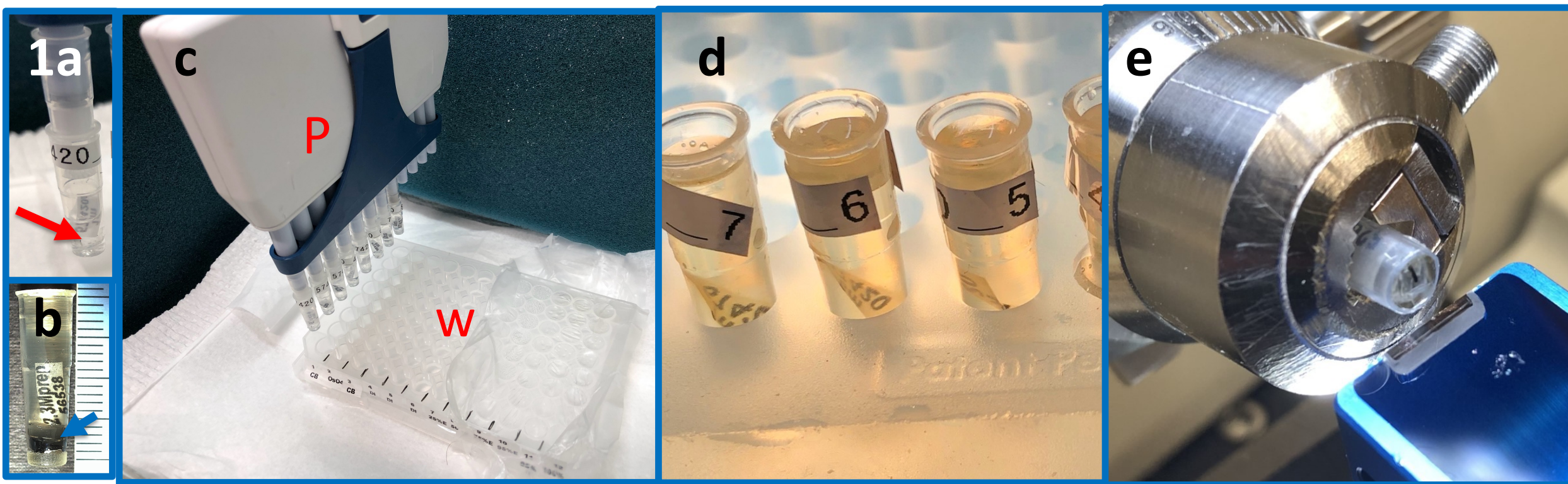


Figure 1a. Oriented ON (arrow) and **1b**, Posterior eye segment (arrow) unoriented in capsules loaded onto a 12-channel electronic pipette (P) with filter couplers (**1c**) for exchanges and mixing of reagents prepared in 96 well plate (W). The pipette was manually moved on boom stand to trigger the programmed liquid aspiration/mixing/displacement through each reagent step. Epoxy resin embedded ON samples oriented in capsules (**1d**) for ultramicrotomy with surface trimming then microtome sectioned using a HISTO diamond knife (**1e**).

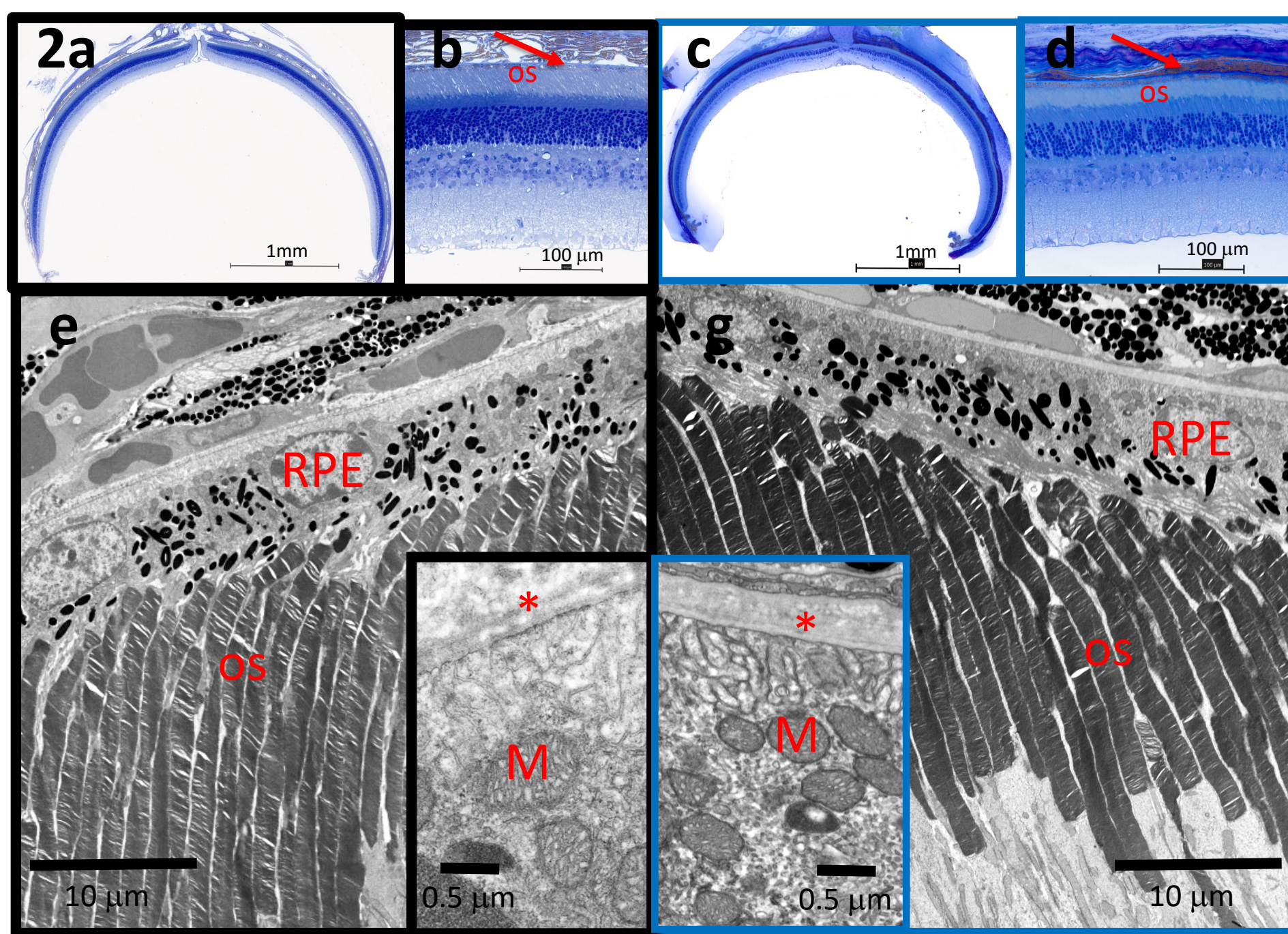


Figure 2. Carousel (**a,b**) & Capsule (**c,d**) epoxy processed & embedded mouse eye posterior segment, semithin section, toluidine blue. Inner and outer retina is preserved with outer segments (OS) and retinal pigmented epithelium (RPE, arrow) intact. Carousel (**e,f**) & Capsule (**g,h**) TEM of outer retina display optimal preservation of cell membranes and organelles (RPE= retinal pigmented epithelium, OS = outer segments, M=mitochondria, *= Bruch's membrane) with similar electron contrast .

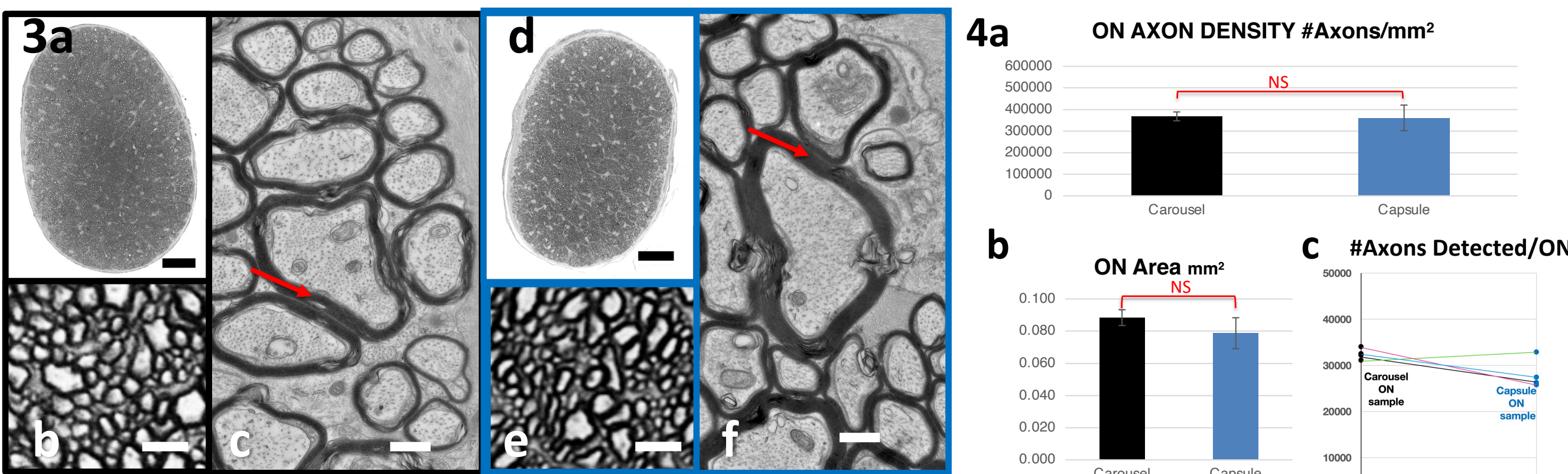


Figure 3. Carousel (**a,b**) and Capsule (**c,d**) epoxy processed & embedded ON. TEM reveals equivalent preservation and contrast of axoplasm, organelles and myelin (arrow). Semithin 1 µm PPD stain (**a,b,d,e**), scale bars= 50 µm (**a,d**), 3 µm (**b,e**), TEM, uranyl acetate & Sato's lead stain, scale bars= 0.5 µm (**c,f**).

Figure 4. Graphs of ImageJ-FIJI AxoNet analysis of ON myelinated retinal ganglion axons revealed no significant difference via T-TEST (NS) in ON axon density (**a**), area (**b**), and #axons detected in each ON between carousel and capsule processed samples. ON samples were obtained within 1mm from each other towards the proximal end.

Results

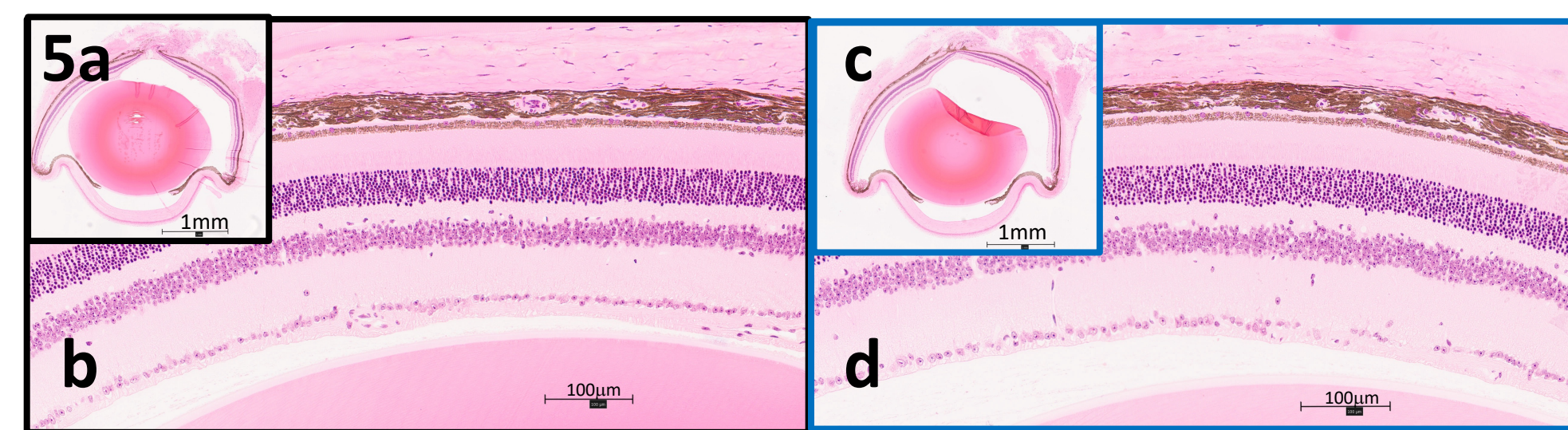


Figure 5. Manual (**a,b**) and Capsule (**c,d**) GMA processed & embedded mouse eyes with identical preservation of retinal layers. 3-um sections, Gill's#2 H&E stained.

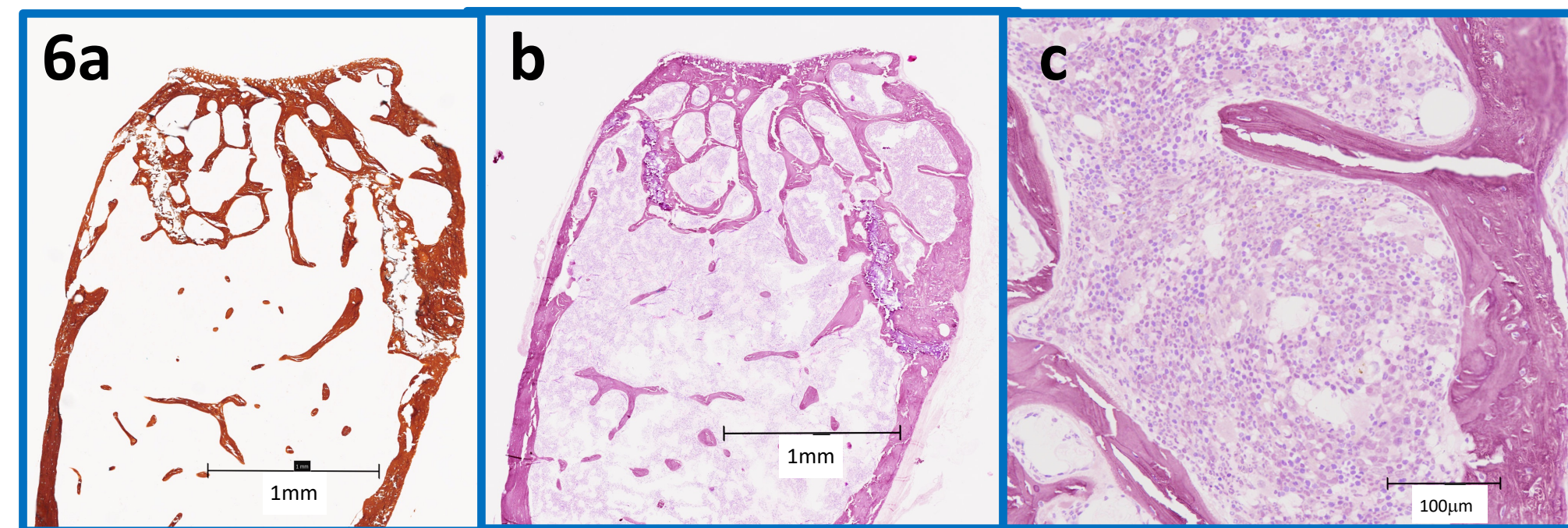


Figure 6. Capsule MBMA processed & embedded mineralized mouse femur head. Marrow tissue and calcium mineral is preserved, 2-um semithin, Von Kossa calcium (**a**), Gill's#2 H&E (**b,c**) stain.

- There were no detectable differences in tissue preservation, staining, or processing related artifacts (e.g. shrinkage, compression) in any of the tissue processed into the resins.
- Enhanced infiltration from the application of capsule processing methods decreased times using minimal solution volumes of 80 uL/solution for each capsule compared to 500 uL/solution for a sample with manual or EMS Lynx 2 carousel processing (2). Capsule processing reagent & solution costs were ~25% the costs of carousel processing.

Conclusion

- Use of capsules with pipette propelled mixing delivers accelerated diffusion of reagents and embedding media into specimens with repeated directed fluid flow that decreases both processing volume and time compared to manual or carousel processing.
- Further protocol development allowing automated capsule tissue processing for histology and electron microscopy applications should be investigated with mouse ocular and other tissue samples.
- A variety of embedding media resins may be used with mPrep/s capsules for histology and electron microscopy tissue processing.

References

- Thomas E Strader *et al.* (2018) Automated Rapid Preparation of Tissue Specimens for TEM Pathology, *Microscopy and Microanalysis*, 24:S1, 1284-1285.
- Philip Seifert (2017) Modified Hiraoka TEM grid staining apparatus and technique using 3D printed materials and gadolinium triacetate tetrahydrate, a nonradioactive uranyl acetate substitute, *Journal of Histotechnology*, 40:4, 130-135.
- Ritch, M. D., *et al* (2020). AxoNet: A deep learning-based tool to count retinal ganglion cell axons. *Scientific reports*, 10:1, 8034.

Acknowledgements, Funding & Disclosure

Supported by NIH National Eye Institute Core Grant P30EY003790. Thanks to the Meredith Gregory Ksander Laboratory for the optic nerve samples, supported by NIH National Eye Institute R01EY032762-02. Special Thanks to the Anatomical Pathology Patient Interest Association for the NSH Travel Poster Scholarship. License agreement between Schepens Eye Research Institute (MGB) & Electron Microscopy Sciences for the Modified Hiraoka TEM Grid Staining Kit IP. The content is solely the responsibility of the presenter and does not necessarily represent the official views of the National Institutes of Health.