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

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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

Step	Reagent	Fluid mix cycles	Time (min)
1	Fix Rinse 1 (Caco. Buffer)	100	3
2	Fix Rinse 2 (Caco. Buffer)	100	3
3	2% OsO4 in Buffer	600	90
4	Rinse 1 (Caco. Buffer)	100	3
5	Rinse 2 (DI water)	100	3
6	2% Uranyl Acetate in DI Water	600	30
7	Rinse 4 (DI water)	100	3
8	25% Ethanol	100	3
9	50% Ethanol	100	3
10	75% Ethanol	100	3
11	95% Ethanol	100	3
12	95% Ethanol	100	5
13	100% Ethanol	100	5
14	100% Ethanol	100	10
15	100% Ethanol	100	3
16	Propylene Oxide (PO)	100	3
17	Propylene Oxide (PO)	100	3
18	25% Epoxy in PO	100	15
19	50% Epoxy in PO	100	15
20	75% Epoxy in PO	100	15
21	100% Epoxy	100	30
22	100% Epoxy	100	30
23	100% Epoxy	(Capsule) 60°C Oven Cure	24 hours
	Steps 1-22		**Time (hours)**
Carousel Processing			**27.2**
Capsule Processing			**4.7**

Table 2. Optic Nerve- Embed812 Epoxy

Step	Reagent	Fluid mix cycles	Time (min)
1	Fix Rinse 1 (Caco. Buffer)	100	3
2	Fix Rinse 2 (Caco. Buffer)	100	3
3	2% OsO4 in Buffer	600	90
4	Rinse 1 (Caco. Buffer)	100	3
5	Rinse 2 (DI water)	100	3
6	Rinse 3 (DI water)	100	3
7	Rinse 4 (DI water)	100	3
8	25% Ethanol	100	3
9	50% Ethanol	100	3
10	75% Ethanol	100	3
11	95% Ethanol	100	3
12	95% Ethanol	100	5
13	100% Ethanol	100	5
14	100% Ethanol	100	10
15	100% Ethanol	100	3
16	Propylene Oxide (PO)	100	3
17	Propylene Oxide (PO)	100	3
18	25% Epoxy in PO	100	15
19	50% Epoxy in PO	100	15
20	75% Epoxy in PO	100	15
21	100% Epoxy	100	15
22	100% Epoxy	100	20
23	100% Epoxy	(Capsule) 60°C Oven Cure	24 hours
	Steps 1-22		**Time (hours)**
Carousel Processing			**26.7**
Capsule Processing			**3.8**

Table 3. Mouse Eye- GMA

Step	Reagent	Fluid mix cycles	Time (min)
1	Rinse (DI water)	100	10
2	25% Ethanol	100	10
3	50% Ethanol	100	10
4	75% MBMA	100	10
5	95% Ethanol	100	10
6	95% Ethanol	100	10
7	100% Ethanol	100	10
8	100% Ethanol	100	10
9	25% GMA	200	15
10	50% GMA	200	15
11	75% GMA	200	15
12	100% GMA	200	15
13	100% GMA	400	15
14	100% GMA Polymerize (External Mold)	(External Mold) RT Cure	24 hours
	Steps 1-13		**Time (hours)**
Manual Processing			**18.1**
Capsule Processing			**2.6**

Table 4. Mouse Femur- MBMA

Step	Reagent	Fluid mix cycles	Time (min)
1	25% Ethanol	100	10
2	50% Ethanol	100	10
3	75% Ethanol	100	10
4	95% Ethanol	100	10
5	95% Ethanol	100	10
6	100% Ethanol	100	10
7	100% Ethanol	100	10
8	100% Ethanol	100	10
9	25% MBMA	200	15
10	50% MBMA	200	15
11	75% MBMA	200	15
12	100% MBMA	400	15
13	100% MBMA	400	15
14	100% MBMA	400	15
15	100% MBMA	400	15
16	100% MBMA w/ catalyst solution	400	15
17	100% MBMA w/ catalyst solution	(External mold) 65°C Oven Cure	24 hours
	Steps 1-16		**Time (hours)**
Manual Processing			**26.8**
Capsule Processing			**3.3**

Methods

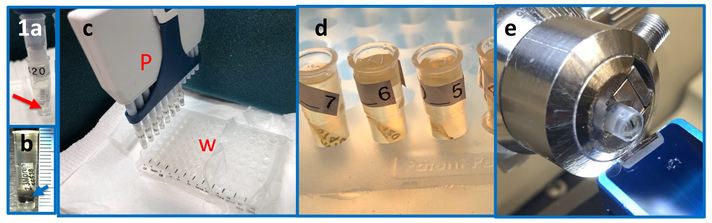

Figure 1a. Oriented ON (arrow) and **1b,** Posterior eye segment (arrow) unoriented in capsules loaded onto a 12-channel electronic pipette (P) with filter couplers (**1c**) for exchanges and mixing of reagents prepared in 96 well plate (W). The pipette was manually moved on boom stand to trigger the 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**).

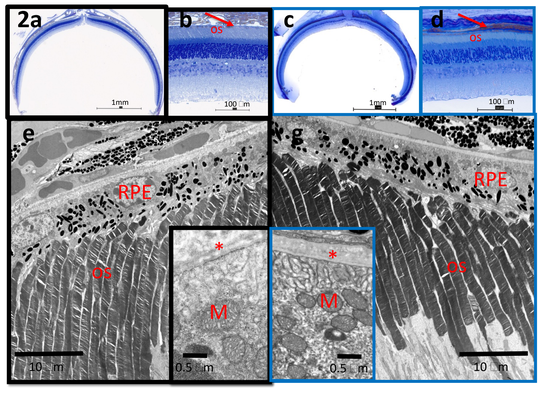

Figure 2. Carousel (**a,b**) & Capsule (**c,d**) **epoxy** processed & embedded mouse eye posterior segment, semithin section, toluidine blue. Inner and outer retina is preserved with outer segments (OS) and retinal pigmented epithelium (RPE, arrow) intact. Carousel (**e,f**) & Capsule (**g,h**) TEM 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 .

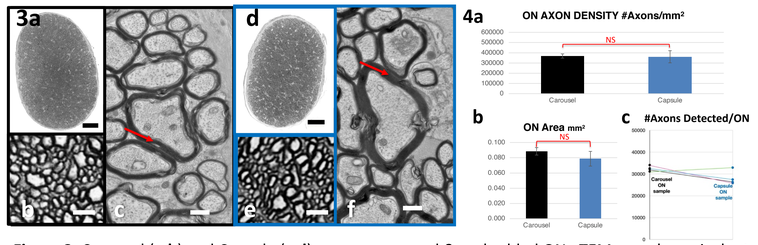

Figure 3. Carousel (**a,b**) and Capsule (**c,d**) **epoxy** processed & embedded ON. TEM reveals equivalent preservation and contrast of axoplasm, organelles and myelin (arrow).
Semithin 1 □m PPD stain (**a,b,d,e**), scale bars= 50 □m (**a,d**), 3□□m (**b,e**), TEM, uranyl acetate & Sato's lead stain,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

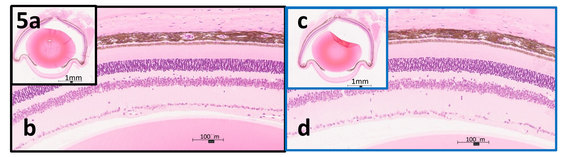

Figure 5. Manual (**a,b**) and Capsule (**c,d**) **GMA** processed & embedded mouse eyes with identical preservation of retinal layers. 3-um sections, Gill's#2 H&E stained.

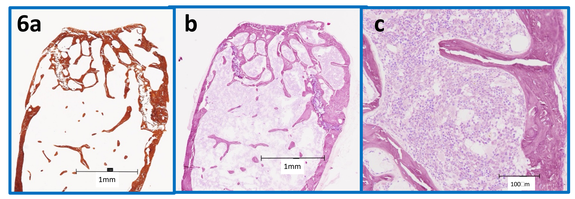

Figure 6. Capsule **MBMA** processed & embedded mineralized mouse femur head. Marrow tissue and calcium mineral is preserved, 2-um semithin, Von Kossa calcium (**a**), Gill's#2 H&E (**b,c**) stain.

- 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

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