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Microwave-assisted Fixation, Labeling and Clearing for Optical Microscopy of Thick Specimens

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Recent innovations in microscope design have increased working distances of optics permitting the collection of subcelluar fluorescence details at depths greater than several millimeters. Sample preparation methodologies have evolved to take advantage of these capabilities, however these protocols take days to months to prepare the tissue for imaging (1). Intact tissue microscopy typically involves fixing and staining, followed by clearing and mounting. Clearing involves treatment with an agent to obviate components that cause light scattering and reduce the ability to image through thick specimens while maintaining morphological and molecular profiles. A wide variety of clearing and mounting media for microscopy have been described in the literature; in several cases the same media are used for both clearing and mounting. Careful attention to clearing and mounting has enabled confocal microscopy of samples greater than 4 mm thick (2). A drawback is that this method requires multiple weeks of clearing time.

Parallel innovation in microwave-assisted processing has been demonstrated to contribute toward accelerating a wide range of processing applications; including live cell imaging, fixations, labeling and embedding, all while preserving probe intensity (3). In this study we have attempted to identify optimal sample preparation, clearing and mounting steps for deep tissue microscopy while significantly shortening the processing time. To this end we tested five commonly used mounting and clearing reagents either hydrophilic or hydrophobic in nature (Table 1). In all cases, we used the same media for clearing and mounting. Porcine skeletal muscle was utilized in this study as a good test of the ability to image deep into a dense, yet uniform, specimen.

Fresh porcine muscle tissue was grossed to roughly 5 mm thick slices and uniform 5 mm punches of tissue were collected. Experimental and control tissue was prepared as in Table 2 utilizing a Ted Pella BioWave Pro microwave using a ColdSpot ™ set to 4^{o}C and vacuum chamber. The sample tempertures were monitored during processing and did not exceed 25^{o}C. The tissue was labeled with 2.0 µM of the far-red excited fluorescent DNA stain To-Pro3 (Life Technologies) to observe nuclei. Once processed, samples were mounted in their respective clearing agent and sealed with No. 1.5 coverslips.

Replicate samples were imaged using a Nikon A1R MP confocal in resonance mode with an Apo LWD 25x/1.1 NA immersion lens and a Nikon A1 confocal microscope using a Plan Fluor 20x/0.75 NA objective. Z-stacks were acquired at appropriate Nyquest sampling steps through 400 microns of the tissue resulting in a voxel size of 160x160x375 nm and 250x250x 875 nm respectively. 3D volumes of these stacks are shown in Figure 1. A depth of approximately 350 µm was reached with microwave-assisted glycerol treatment when observed using the higher NA immersion objective as shown in Figure 2. Significant photobleaching was not observed.

Our findings suggest that microwave-assisted processing increases functional imaging depths on average of 40% greater than parallel treatment samples in the absence of microwaves at identical processing times. Glycerol appears to be an optimal mounting medium when imaging depth is important in an air objective and Spalteholz using an immersion objective. Microwave-assisted processing demonstrates an overall 10-100 fold time-savings while giving improved sample preservation at depth in these

treatments. We will report on the comparisons of microwave-assisted clearing treatments for optimal clearing for both air and immersion optics.

References:

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Table 1. Clearing Reagents

Name	Composition	R.I.	Notes
BABB	33% Benzyl Alcohol: 67% Benzyl Benzoate	1.56	Requires dehydration
*Glycerol	Glycerol	1.47	Dehydration not required
Methyl Salicylate	100% Methyl Salicylate	1.536	Requires dehydration
*Spalteholz (4)	5:3 Methyl Salicylate:Benzyl Benzoate	1.518	Requires dehydration
TDE	97% 2,2'-Thiodiethanol in water	1.515	Dehydration not required

Table 2. Microwave-assisted protocol

Step	Description	Time (on:off:on) min	Power (Watts)	Vacuum (Hg)
1	Fix tissue in 4% paraformaldehyde (in PBS pH 7.2)	10	150	Off
2	Continue fixing tissue in 4% paraformaldehyde	5	250	Off
3	3x PBS-T (0.1% Triton x-100) rinse	2	150	Off
4	0.2 µM ToPro-3 (in PBS pH 7.2)	6:03:06	150	Off
5	3x PBS-T rinse	2	150	Off
6	2x 75% EtOH	5	150	20
7	2x 85%EtOH	5	150	20
7	2x 95% EtOH	5:02:05	150	20
8	3x 100% EtOH	5	150	20
9	5x Clearing solution (repeat until cleared for larger tissues)	6:03:06	150	20
	TOTAL TIME	**< 180 min**		

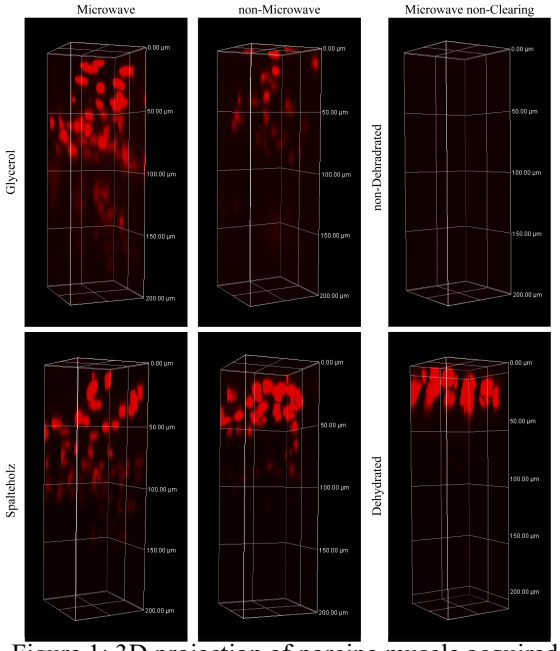

Figure 1: 3D projection of porcine muscle acquired with a NikonA1comparing MW, non-MW and control treatments. 2µM To-Pro3.

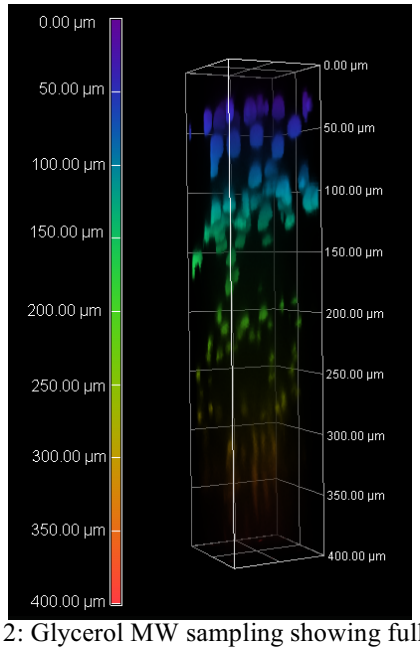

Figure 2: Glycerol MW sampling showing full 400 µm using a Nikon 25x, 1.1NA immersion objective. Colormap corresponds to signal depth of To-Pro3 labeling.

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