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Developing Microwave Techniques for Ultrastructural Double Immuno-Labeling: Pre-Embed DAB and Post-Embed Conjugated Gold.

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The use of the microwave tissue processing has been shown not only to decrease the amount of time needed for processing, but also improve the ultrastructural preservation of the tissue. Traditional methods used for pre-embed immunolabeling take 2-3 days and the use of detergents or freeze thaw can compromise the ultrastructure of the tissue. Many techniques were explored in order to find the optimal balance for double labeling brain tissue and maintaining ultrastructural tissue preservation. All the animals were perfused transcardially with glutaraldehyde fixatives and cut to a thickness of 60 um. The first step was to find the best technique for pre-embed diaminobenzidine (DAB) labeling. Different methods were tried including freeze thaw, Triton X-100, and the microwave. The original microwave method was created from notes and papers provided with the training received during the purchase of the Biowave Pro system from Ted Pella, Inc., and workshops attended prior to purchase. All of the tissue was processed for electron microscopy (EM) using a microwave method previously developed in this lab and flat embedded in EPON/Spurrs resin between 2 ACLAR sheets at 60 degrees C overnight. The area of interest was then cut out of the slices and glued on blank EPON/Spurrs blocks. Thin sections were cut and examined on the electron microscope (JEOL, JEM 1400). It was found that the microwave procedure resulted in less damage to the tissue and the density of DAB labeling was as good as or better than any of the other methods tried (Figure 1). Additional sections were cut and post embed immuno-gold was performed, using 12nm conjugated gold particles, as previously reported [1]. There were no significant differences in the density of immuno-gold labeling following the different DAB pre-embed methods (Figure 2), however, with the double labeling technique, the density of immuno-gold labeling was significantly less compared to carrying out the post-embed labeling alone (data not shown).

Different fixatives were used for perfusing the animals until one was found (1% glutaraldehyde/0.5% paraformaldehyde/0.1% picric acid in 0.1M phosphate buffer, pH 7.3) that gave the best pre-embed DAB and post-embed gold labeling, with only a slight compromise of the density of DAB labeling. After the initial success using a very robust antibody that gave excellent DAB labeling (Figure 3), other antibodies were tried, with some working very well, while others did not. Slight modifications were then made to the original procedure, such as time and wattage of the antigen retrieval and primary antibody steps [2]. The antigen retrieval was found to be a critical step. Also for the antigen retrieval solutions, various antibodies required different pHs (6.0 or 9.0). Some primary antibodies required additional exposure time in the tissue, such that the primary step in the microwave was doubled or occasionally tripled [3].

1. Meshul et. al. *Neuroscience* 88:1-16 (1999)
2. Muñoz et.al. *Journal of Neuroscience Methods* 137:133-139 (2004)

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TABLE 1. EM Processing

Description	time (hr:min:sec)	Watts	Vacuum
OSMIUM ON	0:03:00	100	CYCLE
OSMIUM OFF	0:02:00	0	CYCLE
OSMIUM ON	0:03:00	100	CYCLE
OSMIUM OFF	0:02:00	0	CYCLE
OSMIUM ON	0:03:00	100	CYCLE
RINSE	0:00:40	150	OFF
RINSE	0:00:40	150	OFF
UA	0:02:00	100	CYCLE
UA	0:02:00	0	CYCLE
UA	0:02:00	100	CYCLE
50% ETOH	0:00:40	150	OFF
75% ETOH	0:00:40	150	OFF
95% ETOH	0:00:40	150	OFF
100% ETOH	0:00:40	150	OFF
100% ETOH	0:00:40	150	OFF
PROPYLENE OXIDE	0:00:40	150	OFF
1:1 PO/RESIN	0:03:00	150	CONT
100% RESIN	0:03:00	150	CONT
100% RESIN	0:03:00	150	CONT
100% RESIN	0:03:00	150	CONT
* IF TISSUE IS HAND CUT OR THICKER THAN 100um CONTINUE WITH FOLLOWING STEPS			
100% RESIN	0:03:00	150	CONT
100% RESIN	0:03:00	150	CONT
place tissue in molds or flat embed using ACLAR films place in 60 degree C overnight.			

TABLE 2. Pre-embed Immuno

Description	time (hr:min:sec)	Watts	Vacuum
ANTIGEN RETREIVAL	0:05:00	550	OFF
PBS RINSE	0:01:00	150	OFF
30% HYDROGEN PERIOXIDE	0:01:00	150	OFF
PBS RINSE	0:01:00	150	OFF
PBS RINSE	0:01:00	150	OFF
BLOCKING STEP	0:01:00	150	OFF
PRIMARY PRE-VAC	0:00:10	200	CYCLE
PRIMARY Ab ON	0:02:00	200	CONT
PRIMARY Ab OFF	0:03:00	0	CONT
PRIMARY Ab ON	0:02:00	200	CONT
PBS RINSE	0:01:00	150	OFF
PBS RINSE	0:01:00	150	OFF
SECONDARY Ab ON	0:04:00	200	CYCLE
SECONDARY Ab OFF	0:03:00	0	CYCLE
SECONDARY Ab ON	0:04:00	200	CYCLE
PBS RINSE	0:01:00	150	OFF
PBS RINSE	0:01:00	150	OFF
ABC ON	0:04:00	150	CYCLE
ABC OFF	0:03:00	0	CYCLE
ABC ON	0:04:00	150	CYCLE
PBS RINSE	0:01:00	150	OFF
PBS RINSE	0:01:00	150	OFF
DAB ON BENCH ~10min Stop reaction by putting tissue in PBS. Put tissue in change of PBS until ready to process for EM			

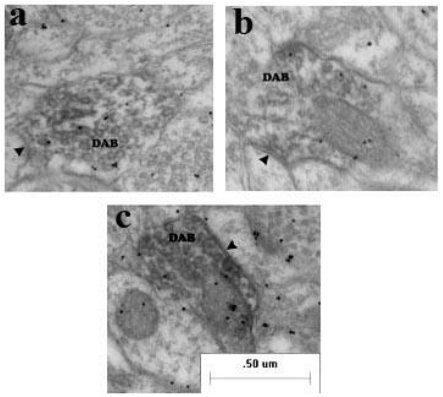

Fig.1. Methods of pre-embed DAB immunohistochemistry (a)freeze-thaw,(b)Triton-X-100 and (c)microwave

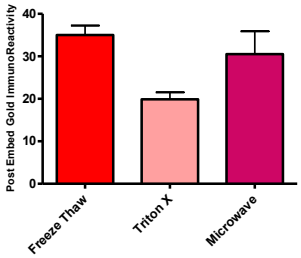

Fig.2. Density of glutamate immuno-gold labeling using different methods of DAB pre-embed immunohistochemistry.

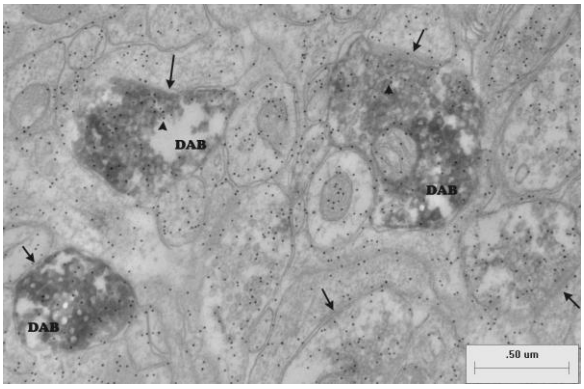

Fig.3. Pre-embed DAB labeled (VGLUT1) and post-embed gold label (glutamate).

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