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Apr 04, 2022

Preparation of Biological Tissues for Serial Block Face Scanning Electron Microscopy (SBEM) V.2

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dx.doi.org/10.17504/protocols.io.36wgq7je5vk5/v2

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This protocol was designed to enhance signal for backscatter electron imaging of epoxy embedded mammalian tissue at low accelerating voltages (1-3 keV). However, it can easily be adapted for use with tissues from other species, tissue culture cells, plants and microbial cells by adjusting the buffer strength and the duration of relevant steps. This combinatorial heavy metal staining protocol employs a battery of contrasting steps after primary aldehyde fixation including: ferrocyanide reduced osmium tetroxide post fixation, thiocarbohydrazide-osmium liganding (OTO) and subsequent uranyl acetate and en bloc lead aspartate staining. Calcium chloride is included in a number of steps to enhance membrane preservation and staining. This protocol was designed primarily to emphasize the contrast of membranes. Many other contrasting agents may be included to increase staining of other cellular and extracellular constituents.

DOI

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https://ncmir.ucsd.edu/sbem-protocol

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Thomas J. Deerinck, Eric A. Bushong, Mark H. Ellisman, Andrea Thor 2022. Preparation of Biological Tissues for Serial Block Face Scanning Electron Microscopy (SBEM). **protocols.io**
https://dx.doi.org/10.17504/protocols.io.36wgq7je5vk5/v2
NCMIR

NIH/National Institute of Neurological Disorders and Stroke
Grant ID: U24NS120055

NIH/National Institute of General Medical Sciences
Grant ID: R24GM137200

biological specimens, serial blockface scanning electron microscopy , biological tisuses , UCSD, NCMIR

protocol ,

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Specimen Mounting

The following procedure is used to mount specimens to minimized specimen charging.

Small pieces of resin embedded tissues are mounted on aluminum specimen pins (Gatan, Pleasanton, CA) using cyanoacrylate glue. The blocks are faced and precision trimmed with a glass knife to a square approximately 1.0 mm x 1.0 mm so that tissue is exposed on all four sides. Silver paint (Ted Pella) is used to electrically ground the exposed edges of the tissue block to the aluminum pin taking care not to get the paint on the block face or edges of embedded tissue that will ultimately be sectioned. The entire surface of the specimen is then sputter coated with a thin layer of gold/palladium. After the block is surfaced with the 3View ultramicrotome to remove the top layer of gold/palladium, the tissue can be imaged using BSE mode. The coating on the edges of the block plays an important role in reducing charging and does not interfere with the sectioning process. Some charging may be noted in cell nuclei and in blood vessels under high vacuum conditions and if needed can be eliminated by using the variable pressure mode.

Addendum: The use of double distilled water (ddH2O) in making solutions is highly recommended.

Durcupan resin epoxy in liquid form and paraformaldehyde are carcinogens, please wear proper PPE.

Sodium Cacodylate contains arsenic, therefore it is toxic. Wear proper PPE.

Uranyl acetate and glutaraldehyde are toxic, wear proper PPE.

Osmium tetroxides are very powerful oxidizers, they must be used in the fume hood.

1 Animals are anesthetized and perfused with normal Ringer's solution (see protocol for Ringer's solution) containing xylocaine (0.2 mg/ml) and heparin (20 units/ml) for 2 minutes at 35°C followed by 0.15M cacodylate buffer (Product No. 18851, Ted Pella Inc.) pH 7.4 containing 2.5% glutaraldehyde (Product No: 18426, Ted Pella, Inc.)(Electron Microscopy Sciences, Hartfield, PA), 2% paraformaldehyde (Product No. 19202, Electron Microscopy Sciences) (see protocol for creating fixative solution) with 2mM calcium chloride at 35°C for 5 minutes.

2 Target tissues are then removed and fixed for an additional 2-3 hours on ice in the same solution.

3 Some tissues such as brain should be cut into 80-100 micron thick vibratome sections in ice-cold 0.15M cacodylate buffer containing 2mM calcium chloride. Other tissues may be cut into small (<2mm x 2mm) pieces with a razor blade.

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4 Tissues are washed 5 x 3 minutes in cold cacodylate buffer containing 2mM calcium chloride.

5 Right before use, a solution containing 3% potassium ferrocyanide in 0.3M cacodylate buffer with 4mM calcium chloride is combined with an equal volume of 4% aqueous osmium tetroxide (EMS). The tissues are incubated in this solution for 1 hour, on ice.

6 While the initial osmium incubation (step 5 above) is occurring prepare the following thiocarbohydrazide (TCH) solution (Product No. T1136-25G, VWR International). This reagent needs to be fresh and available right at the end of step 5. Add 0.1 gm thiocarbohydrazide to 10 ml ddH_2O and place in a 60° C oven for 1 hour, (agitate by swirling gently every 10 minutes to facilitate dissolving). Filter this solution through a 0.22 um Millipore syringe filter right before use.

7 At the end of the first heavy metal incubation described in Step 5 (before adding the TCH) the tissues are washed with ddH_2O at room temperature 5 x 3 minutes (~15 minutes total).

8 Tissues are then placed in the 0.22 micron Millipore filtered TCH solution for 20 minutes, at room temperature.

9 Tissues are then rinsed again 5 x 3 minutes in ddH_2O at room temperature and thereafter placed in 2% osmium tetroxide (Product No. 19190, Electron Microscopy Sciences) (NOT osmium ferrocyanide) in ddH20 for 30 minutes, at room temperature.

10 Following this second exposure to osmium the tissues are washed 5 x 3 minutes at room temperature in ddH_2O then placed in 1% uranyl acetate (Product No. 22400 Electron Microscopy Sciences) (aqueous) and left in a refrigerator (~4°) overnight.

11 The next day, en bloc Walton's lead aspartate staining is performed. First, prepare an aspartic acid stock solution by dissolving 0.998 gm of L-aspartic acid (Sigma-Aldrich) in 250 ml of ddH_2O Note: the aspartic acid will dissolve more quickly if the pH raised to 3.8. This stock solution is stable for 1-2 months if refrigerated. To make the stain dissolve 0.066 gm of lead nitrate in 10 ml of aspartic acid stock and pH adjusted to 5.5 with 1N KOH. The lead aspartate solution is placed in a 60°C oven for 30 minutes (no precipitate should form). The tissue is washed 5 x 3 minutes in ddH_2O at room temperature and then placed in the lead aspartate solution and then returned to the oven for 30 minutes.

12 The tissues are washed 5 x 3 minutes in room temperature ddH_2O and dehydrated using ice-cold solutions of freshly prepared 20%, 50%, 70%, 90%, 100%, 100% ethanol (anhydrous), 5

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minutes each, then placed in anhydrous ice-cold acetone and left at room temperature for 10 minutes.

13 Tissues are placed in room temperature acetone for 10 minutes. During this time, Durcupan ACM resin (EMS) is formulated by weight as follows: 11.4 gm part A (Product No. 102384658 Sigma Aldrich), 10 gm part B (Product No. 102418273 Sigma Aldrich), 0.3 gm part C (Product No. 102131679 Sigma Aldrich) and 0.05-0.1 gm part D (Product No. 101886488 Sigma Aldrich), yielding a hard resin when polymerized. The resin is mixed thoroughly samples are placed into 25% Durcupan:Acetone for 2 hours, then into 50% Durcupan:Acetone for 2 hours and 75% Durcupan:Acetone for 2 hours.

14 Tissues are placed in 100% Durcupan overnight then into fresh 100% Durcupan for 2 hours. Tissue sections are then mounted between liquid release agent-coated glass slides (EMS) and tissue pieces are embedded in a thin layer of fresh resin in an aluminum weigh boat and place in a 60°C oven for 48 hours.

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