PREPARATION OF BIOLOGICAL SPECIMENS FOR

SERIAL BLOCK FACE SCANNING ELECTRON MICROSCOPY

This sample preparation scheme is based on Ellisman[1] protocol to enhance signal for backscatter electron imaging of epoxy embedded mammalian tissue at low accelerating voltages. It is designed primarily to emphasize the contrast of membranes. Many other contrasting agents may be included to increase staining of other cellular and extracellular constituents. This protocol 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.
It is very important to ensure samples bigger than 800 μm^3 are adequately infiltrated with metals and resins by increasing the duration of these steps. The smaller and/or thinner sample size significantly improves the infiltration. Working with vibratome sections of < 200 µm is highly recommended.

The use of double distilled water (ddH_2O) in making solutions is highly recommended.

1. Fix tissue in your preferred fixative/buffer, e.g. 2.5% glutaraldehyde, 2% formaldehyde (freshly made) with 2mM calcium chloride in 0.15M cacodylate buffer (pH 7.4) for 2-3 hours on ice.
2. Some tissues such as brain should be cut into 80-100 µm thick vibratome sections in ice-cold 0.15M cacodylate buffer containing 2mM calcium chloride. Other tissues may be cut into small (<0.5 mm x 0.5 mm x 0.5 mm) pieces with a razor blade.
3. Rinse samples in cold cacodylate buffer containing 2mM calcium chloride for 5 x 3 min.
4. Right before use, mix equal volumes of 4% osmium tetroxide and 3% potassium ferrocyanide in 0.3M cacodylate buffer with 4mM calcium chloride.
   Incubate samples in this solution for 1 h, on ice.
5. During the initial osmium incubation (step 4), prepare thiocarbohydrazide (TCH) solution. This reagent needs to be fresh and available right at the end of step 4 (see page 2).
6. Before incubation with TCH solution, wash samples with ddH_2O for 5 x 3 min at RT.
7. Incubate samples in the TCH solution (0.22 µm Millipore filtered) for 20 min at RT.
8. Rinse samples in ddH_2O for 5 x 3 min at RT.
9. Incubate samples in 2% osmium tetroxide in ddH_20 (NOT ferrocyanide reduced osmium) for 30 min at RT.
10. Rinse samples in ddH_2O for 5 x 3 min at RT.
11. Incubate samples in 1% uranyl acetate and leave in a refrigerator (~4°C) overnight.
12. Next day, rinse samples in ddH_2O for 5 x 3 min at RT followed by *en bloc* lead aspartate (see page 2) staining for 30 min at 60°C (0.02 M lead nitrate in 0.03 M sodium aspartate, pH 5.5; Walton, j. Histochem Cytochem., 1979).
13. Wash samples with ddH_2O for 5 x 3 min at RT.
14. Dehydrate samples with graded series of ethanol (freshly prepared ice-cold solutions 20%, 50%, 70%, 90%, 100%, 100% ethanol (anhydrous)), 2x7 min each step, then place in anhydrous ice-cold acetone for 10 min at RT.
15. Tissues are placed in RT acetone for 10 min.

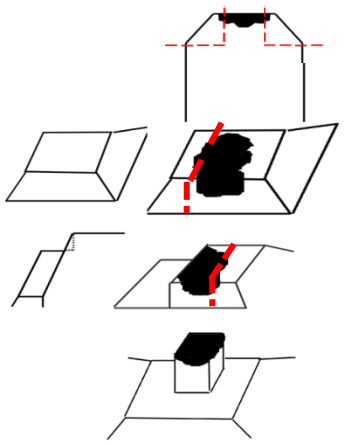
16. Infiltrate samples with 25% Durcupan:acetone for 2-3 h at RT.
17. Infiltrate samples with 50% Durcupan:acetone for 2-3 h at RT.
18. Infiltrate samples with 75% Durcupan:acetone for 3 h at RT.
19. Infiltrate samples with 100% Durcupan overnight on shaker.
20. Next day, continue further infiltration with fresh 100% Durcupan for 6 h.
21. Embed samples in fresh resin using flat embedding molds and polymerize for 48 h at 60°C oven.
    Vibrotome sections can be mounted between Liquid Release Agent-coated glass slides (EMS) and tissue pieces can be embedded in a thin layer of fresh resin in an aluminum weight boat, polymerize for 48 h at 60°C oven.

EPON HARD CAN ALSO BE USED INSTEAD OF DURCUPAN RESIN

Specimen Trimming and Mounting

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

1. Using an ultramicrotome trim the sample either into a square or a rectangle, exposing the tissue on all 6 sides of the block (preferred dimensions of 500 µm x 500 µm x 500 µm or less).
2. Once the block is trimmed, cut it off from the rest of the resin block using a razor blade- be careful that the sample doesn't jump or fly away.
3. Mount exposed surface of the sample onto aluminum specimen pin using using cyanoacrylate glue and/or silver paint. One may also use a two component silver conductive epoxy.
4. Proceed with facing the unexposed part of the block until sample is exposed.
   Around the edges of the sample, apply silver paint. Take care not to get the paint on the block face or edges of embedded tissue that will ultimately be sectioned.
   Silver paint is used to electrically ground the exposed edges of the tissue block to the aluminum pin.
5. Sputter coat the entire surface of the specimen with a thick layer of gold/palladium.
6. Using an ultramicrotome, face the blockface to remove the top layer of gold/palladium coating.
   The coating on the edges of the block plays an important role in reducing charging and does not interfere with the sectioning process.
   Excess of silver paint or epoxy can be removed by trimming the sample using an ultramicrotome.



Thiocarbohydrazide (TCH) solution
This reagent needs to be fresh and available right at the end of step 4.
Add 0.1 g thiocarbohydrazide to 10 ml ddH_2O and place in a 60°C oven for 1 h, (agitate by swirling gently every 10 min to facilitate dissolving). Filter this solution through a 0.22 µm Millipore syringe filter right before use.

En bloc Walton's lead aspartate
Prepare an aspartic acid stock solution by dissolving 0.998 g of L-aspartic acid in 250 ml of ddH_2O. Note: the aspartic acid will dissolve more quickly if the pH is 3.8. This stock solution is stable for 1-2 months if refrigerated. To make the stain dissolve 0.066 g 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).

Resins for Serial Block Face-SEM

Durcupan ACM resin hard (EMS)
Mix in this order, stirring thoroughly after each component

- 11.4 g component A
- 10 g component B
- 0.05-0.1 g component D
- 0.3 g component C
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Epon resin hard (modified from H. Horstmann/W. Denk)

- 37 ml Glycidether (e.g. Roth or Embed 812, EMS)
- 25 ml DDSA
- 20 ml MNA
- 1.3 ml DMP-30

Reference:

[1]Thomas J. Deerinck, Eric A. Bushong, Andrea Thor and Mark H. Ellisman
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