Processing tissue for serial block-face SEM

Reference:
1 NCIMIR Methods For 3D EM: A New Protocol For Preparation of Biological Specimens for Serial Block Face Scanning Electron Microscopy (7-1-10)
Thomas J. Deerinck, Eric A. Bushong, Andrea Thor and Mark H. Ellisman
2 Biologicke Centrum Av CR, Parazitologicky ustav, Czech Republic; Thermo Fisher

Protocol:
Day 1:

1. Fixation is critical for optimal results. Use freshly prepared SBFSEM fixative (2.5% glutaraldehyde, 2% formaldehyde, in 0.15M cacodylate buffer with 2mM CaCl₂). For tissues, dissect quickly into no greater than 2mm³ pieces and immediately immerse in fixative. For cells, submerge in fixative as soon as possible after treatment. Store at 4°C for a minimum of 1 hr. We prefer processing tissue in small, clear, glass sample vials (Fisher #0333520AA).

2. Rinse in 0.15M cacodylate buffer containing 2mM calcium chloride 4 x 3min.

3. Incubate in fresh 2% osmium tetroxide in 0.15M cacodylate buffer, pH 7.4. for 1.5 hr RT, rotating

4. Prepare 1% thiocarbohydrazide (TCH) solution. This reagent needs to be fresh and available right at the end of step 6.

5. WITHOUT a wash step, remove the osmium tetroxide and MW samples in fresh 2% osmium tetroxide in 2.5% potassium ferrocyanide in 0.1M cacodylate buffer for 1.5 hr RT, rotating

6. Rinse in nH₂O 4 x 3min.

7. Incubate in filtered TCH solution (0.22µm Millipore filtered) for 45min at 40°C.

8. Rinse in nH₂O 4 x 3min.

9. Incubate for 1.5h at RT, rotating, in 2% fresh osmium tetroxide in nH₂O

10. Rinse in nH₂O 4 x 3min at RT.

11. Incubate in 1% aqueous uranyl acetate, 4°C overnight.

Day 2:

12. Move samples to 50°C oven for 1 hr in same UA solution.

13. Rinse in nH₂O 4 x 3min at RT.

14. Incubate in Walton’s Lead aspartate for 1 hr at 50°C.

15. Rinse in nH₂O 4 x 3min at RT.
16. Dehydrate samples at RT with gentle agitation as follows:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% EtOH</td>
<td>1 x 10min</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>1 x 10min</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>1 x 10min</td>
</tr>
<tr>
<td>90% EtOH</td>
<td>1 x 10min</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1 x 10min</td>
</tr>
<tr>
<td>Acetone</td>
<td>2 x 10min</td>
</tr>
</tbody>
</table>

If the tissue is in a plastic container, DO NOT use acetone.

17. Infiltrate as follows: Remove acetone and replace with the respective Durcupan resin: acetone ratio for the designated amount of time, at RT, as follows below:

<table>
<thead>
<tr>
<th>Concentration of resin in acetone</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>33%</td>
<td>0.5 hr rotating</td>
</tr>
<tr>
<td>50%</td>
<td>1 hr rotating</td>
</tr>
<tr>
<td>75%</td>
<td>2 hr rotating</td>
</tr>
<tr>
<td>100%</td>
<td>Overnight</td>
</tr>
</tbody>
</table>

If the tissue is in a plastic container, DO NOT use acetone, substitute ethanol instead.

**Day 3:**

18. Remove samples from 100% resin in processing vials and transfer to fresh 100% Durcupan resin in flat embedding molds. Orient tissue as desired and polymerize for 24 hr minimum in 60°C oven.

**Day 4: Preparation of stub**

19. Using a sharp razor blade, cut tissue from block into a small enough piece to fit on center of 8 mm aluminum stub (remove any excess resin). Generally ~1 mm³.

20. Mount the piece of tissue using Epo-Tek silver epoxy (Ted Pella #16014), cure epoxy in Thermo drying oven for 3 hr.

21. Using a razor blade, trim block into tower or pyramid shape with very clean edges. This should be no greater than 0.3 mm x 0.3 mm x 1.0 mm tall.

22. Use an ultramicrotome and diamond knife to face off tower of tissue. Collect thin sections at this time for reference if necessary.

23. Sputter coat the entire surface of the specimen with a thick layer (20-30nm) of gold/palladium.

24. Use an ultramicrotome and diamond knife to face off tower of tissue to remove the top layer of gold/palladium coating.

25. Stub is ready to go into the Volumescope.

**Reagents:**
cacodylate buffer (0.3M stock)
sodium cacodylate tri-hydrate (EMS #12300 FW214) 16.05g  
H₂O 250ml  
Adjust pH to 7.4

0.3M Cacodylate buffer with 4mM calcium chloride
calcium chloride anhydrous (Sigma C-1016 FW111) 0.11g  
0.3M cacodylate stock 250ml

SBFSEM Fixative: (2.5% glutaraldehyde, 2% formaldehyde, in 0.15M cacodylate buffer with 2mM CaCl₂)
25% glutaraldehyde (EMS #16220) 2.5ml  
16% paraformaldehyde (EMS #15710) 3.1ml  
0.3M cacodylate stock + 4mM CaCl₂ 12.5ml  
H₂O 6.9  
Total = 25ml

2% osmium tetroxide in 0.15M cacodylate buffer + 2mM CaCl₂
powdered osmium 2g  
0.3M Cacodylate buffer with 4mM calcium chloride 50ml  
H₂O 50ml

2% osmium + 2.5% potassium ferracyanide in 0.15M cacodylate buffer
KFeCN (Sigma # P-8131) 0.05g  
2% OsO₄ in 0.15M cacodylate buffer + 2mM CaCl₂ 2.0ml

1% thiocarbohydrazide (TCH)
Thiocarbohydrazide (EMS #21900) 0.1g  
H₂O 10ml  
Place into 15ml conical tube and heat to 40°C for 30-60min, until the solid is in solution and there is no more TCH precipitate, mix occasionally. Filter using syringe filter 0.2μM prior to use

2% osmium tetroxide in H₂O
powdered osmium 2g  
H₂O 100ml

2% uranyl acetate in H₂O
2% uranyl acetate (EMS cat # 22400-2)  
Use straight from vendor stock

Aspartic acid stock solution
L-aspartic acid (Sigma #?) 0.998g  
H₂O 250ml  
Raise the pH to 3.8; store at 4°C.

Walton’s Lead aspartate stain
Lead nitrate (MP Biomedicals #155180) 0.066g  
Aspartic acid stock solution (Sigma #A-8256) 10ml  
Adjust pH to 5.5 with 1N KOH. Place in 60°C oven for 30min.
## Durcapan Resin (EMS #14040)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part A-Bisphenol-A-Epoxy</td>
<td>10 ml</td>
<td>11.4 g</td>
</tr>
<tr>
<td>Part B-964 Hardener</td>
<td>10 ml</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Part C-960 Accelerator</td>
<td>0.3-0.4 ml</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Part D-Dibutyl phthalate</td>
<td>0.1-0.2 ml</td>
<td>0.05-0.1 g</td>
</tr>
</tbody>
</table>

## Embed 812 hard resin

<table>
<thead>
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<th>Component</th>
<th>Volume</th>
</tr>
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<tbody>
<tr>
<td>Embed 812</td>
<td>31.4 ml</td>
</tr>
<tr>
<td>DDSA</td>
<td>9.3 ml</td>
</tr>
<tr>
<td>NMA</td>
<td>20.5 ml</td>
</tr>
<tr>
<td>DMP-30</td>
<td>0.8 ml</td>
</tr>
</tbody>
</table>

Mix thoroughly after the addition of each component, degas under vacuum, use fresh.