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Preparation of Cultured Cells for Serial Block Face Scanning Electron Microscopy (SBEM)

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We use this protocol and it's working

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Abstract

Serial block-face scanning electron microscopy (SBEM) is a 3D EM method that allows volume reconstruction of biological samples. This protocol has been adapted for processing of cells in culture grown on glass-bottom dishes.

Troubleshooting

Safety warnings

Fixatives and electro-microscopy stains are extremely hazardous and should be prepared and handled in a fume hood. Gloves and protective eyewear should be used at all times.

1 Cells are fixed in glutaraldehyde (2.5% in 0.1M sodium cacodylate) for 60 minutes (room temperature to ice).

2 Cells are washed 5 × 2 minutes in cold cacodylate buffer containing 3mM calcium chloride.

3 Right before use, a solution containing 3% potassium ferrocyanide in 0.2M cacodylate buffer with 6mM calcium chloride is combined with an equal volume of 4% aqueous osmium tetroxide (EMS). The cells are incubated in this solution for 30 minutes on ice.

4 While the initial osmium incubation (step 3 above) is occurring, prepare the following thiocarbohydrazide (TCH) solution. This reagent needs to be fresh and available right at the end of step 3. Add 0.1 gm thiocarbohydrazide (Ted Pella) to 10 ml ddH_2O and place in a 60° C oven for 30 minutes, (agitate by swirling gently every 10 minutes to facilitate dissolving). Filter this solution through a 0.22 um Millipore syringe filter right before use.

5 At the end of the first heavy metal incubation described in Step 3 (before adding the TCH), the cells are washed with ddH_2O at room temperature 5 × 2 minutes.

6 Cells are then placed in the 0.22 micron Millipore filtered TCH solution for 10 minutes at room temperature.

7 Cells are then rinsed again 5 × 2 minutes in ddH_2O at room temperature, and thereafter placed in 2% osmium tetroxide (NOT osmium ferrocyanide) in ddH_2O for 10 minutes at room temperature.

8 Following this second exposure to osmium the tissues are washed 5 × 2 minutes at room temperature in ddH_2O then placed in 2% uranyl acetate (aqueous) and left in a refrigerator (~4°) overnight.

9 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 cells are washed 5 × 2 minutes in ddH_2O at room temperature and then placed in the lead aspartate solution and then returned to the oven for 10 minutes.

10 The cells are washed 5 × 2 minutes in room temperature ddH_2O and dehydrated using ice-cold solutions of freshly prepared 20%, 50%, 70%, 90%, 100%, 100% ethanol

(anhydrous), 3 minutes each, then placed in anhydrous 100% ethanol at room temp, 3min.

11 Durcupan ACM resin (EMS) is formulated by weight as follows: 11.4 g part A, 10 g part B, 0.3 g part C and 0.05-0.1 g part D, yielding a hard resin when polymerized. The resin is mixed thoroughly samples are placed into 50% Durcupan:ethanol for 30 minutes, then into 100% Durcupan overnight. The following day: 3 changes of 100% Durcupan for 1-2 hours.

12 A small amount of resin is placed over the cells and a square piece of aclar (large enough to cover the hole) is placed over the top opening to create a flat surface and placed in a 60° oven for 48 hours.