FIXING AND EMBEDDING FOR TEM

(Hugh Dickinson's Protocol)

INTRODUCTION

The procedure set out below is for fixing and embedding plant material which is not heavily cuticularized or otherwise impenetrable. The rationale behind these steps is the fixation of proteins by formaldehyde, which is volatile and penetrates the material well and glutaraldehyde which penetrates less well but preserves structure better. After fixation in these aldehydes, material is subject to post-fixation step using osmium tetraoxide. This forms hexameric osmium complexes that both preserve structure and produce the beginnings of some contrast for the electron microscope. Fixation is carried out in an aqueous environment, but the plastics used for embedding prior to sectioning are almost exclusively covalent. For this reason the material has to be dehydrated in a water/acetone series. Infiltration with resin is also a problem because it is very viscous and, even if miscible with the solvent, will not penetrate the material. The tissue is therefore infiltrated, often over a period of days, with resin/solvent mixes that introduce increasing concentrations of resin into the material. Finally the resin is polymerised in the oven.

While the steps in this process are relatively straightforward, some are hazardous. The fixatives, by their nature, are aggressive and will fix human tissue just as well as plant material. All handling of fixatives and resins must be carried out using gloves and, where appropriate, safety spectacles. **The handling of osmium is particularly hazardous and no-one should attempt this without prior instruction**. Likewise the vacuum infiltration stage is potentially dangerous and should not be attempted without prior instruction.

THE PROCESS

1. Make 0.1M phosphate buffer, pH 7.0
   (61 ml of stock solution A & 39 ml of stock solution B)

2. In the fume hood, weigh 4g of paraformaldehyde and add 20 ml of distilled water and 1 pellet of NaOH in a 100 ml glass beaker. Heat

the solution in a stirrer at 60°C (do not exceed temperature) until dissolved.

3. Cool solution by placing in ice water.

4. Add 12 ml of a 25% solution of glutaraldehyde and adjust the pH to 7.0 by adding H_2SO_4 (1M first and 0.1M once it has reached pH 10.0).

5. Pour the solution into a flask. Add 50 ml 0.1M of the previously prepared phosphate buffer. Make the volume up to 100 ml with water and check the pH.

6. Mix thoroughly and cover with parafilm; store cold.

7. In fumehood dissect the material under the surface of a drop of fixative and place in small screw-top vials. Label carefully with a code which will enable you to recognise similar-looking resin blocks!

8. **IMPORTANT – YOU MUST FIRST BE INSTRUCTED IN THIS PROCEDURE!** Vacuum infiltrate the material. Place tubes (without their caps) in a vacuum infiltrator and run for as many cycles as necessary for the material to sink in the fixative

9. Replace the fixative, and leave material to fix for 3-5 hours (up to 10hrs normally does not cause damage).

10. Carefully remove the fixative solution with a disposable plastic pipette (stretch tip) and discard it appropriately.

11. Wash the samples in two 10 min changes of 0.05 M phosphate solution (dilute the previously prepared solution by half).

12. Fill beaker with industrial methylated ethanol and keep aside to soak pipettes contaminated with osmium tetraoxide.

13. **IMPORTANT – YOU MUST FIRST BE INSTRUCTED IN THIS PROCEDURE**! Drain the specimens and add 0.5 ml water and 1 ml 2% aqueous osmium tetraoxide (**OsO4 is extremely toxic and very volatile!!**). Leave between 1 and 2 hours depending on the tissue (for buds almost 2 hours). OPTIONAL STEP: You can add 0.5ml 0.1M

buffer instead of the water (to avoid osmotic damage); this may, however, cause contamination.

14. **IMPORTANT – YOU MUST FIRST BE INSTRUCTED IN THIS PROCEDURE**! Remove the osmium tetraoxide and discard appropriately. Rinse the material in two, 10 min changes of distilled water.

15. In order to dehydrate the samples, perform a series of acetone washes (ethanol is not used because it reacts with osmium tetraoxide and forms a black precipitate). Acetone series; 25. 50, 75, 95 and 100% (3 times with pure, dry acetone). Leave in each mixture for 30mins. **IT IS ESSENTIAL THAT THE MATERIAL IS NEVER ALLOWED TO DRY OUT DURING THIS PROCESS** (acetone is very volatile). If placed in a rotator in the fume hood, the samples are continually exposed to fresh acetone/water mixture (but they may stick to the tube walls). The samples may be left overnight in the acetone solutions. At the 75% acetone stage, write the code for each sample in pencil on a very small piece of paper – place in the appropriate tubes and handle as the material.

16. Embedding in resin. Use **TAAB 812 Resin Premix Kit – Hard Plus T027/1** (not TAAB Embedding Resin!). At room temperature, mix the contents of the two bottles (as directed by the instruction sheet) add accelerator and shake very well for 5 minutes. Resins are carcinogenic – avoid contact with skin, wipe the bottles and check gloves. Resins need not be handled in the fume cupboard.

17. To move from an ionic (water) to a covalent (resin) environment, the material is passed through a resin/acetone dilution series. Using the 100% resin mix, make appropriate resin/dry acetone mixtures (25%, 50%, 75%) in a 50 ml Falcon tube immediately prior to use. Ensure the resin mix is a room temperature prior to use, and return to the fridge after using. Partially drain the acetone from the material (**MUST NOT DRY OUT**) and add a few mls of acetone/resin mixture. Place on the rotator for at least 4 hours for each acetone/resin mix.

18. Add 2.5 ml of the full resin mixture to each tube, place on the rotator for at least 5 hours (overnight is OK). Repeat this operation.

19. Pour resin mix to a depth of 3mm in an aluminium tray (one for each tube). Place the tubes containing the samples in the oven for 10 min to make the resin more liquid. With a wooden cocktail stick (with the end cut) or a fresh pipette carefully remove the material and evenly distribute on the base of the tray. Align the labels so they may be seen.

20. In the fume hood, bake the trays at 60 degrees in a purpose-made oven for 24 hours. Allow to cool. These are now ready to saw out samples and start sectioning.

Stock solutions

Phosphate buffers

A: 0.1M solution of dipotassium hydrogen phosphate
17.418g K_2HPO4 in 1000 ml water*

B: 0.1M solution of potassium dihydrogen phosphate
13.609g KH_2PO4 in 1000 ml water*

- For pH 7.0

'Dry Acetone'

Fill 100 ml Durian bottle with acetone, add 3-4 mm depth of molecular sieve type 3A to bottom (crystalline potassium alumino-silicate). Shake and leave for an hour.

Osmium Tetraoxide

Order from Agar Scientific R1015 10 × 0.1g vials.

To make solution carefully wrap tissue around vial and snap off top. Put the whole bottom piece of broken vial into a dedicated glass bottle with glass stopper. Add water required to make 2% solution for the specimens you have and shake. (To 0.1g Osmium we added 5 ml water and this was enough for 5 specimens).