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STANDARD (Non-Microwave) SPECIMEN PREPARATION PROTOCOLS FOR SEM, TEM AND IMMUNOLABELING

Use appropriate protective clothing for all steps!

For transmission electron microscopy (TEM), specimens should be cut into very small pieces, preferably 1mm or less on a side. This should be done within in fixative within seconds after harvesting tissue and pieces may be transferred to a small container (such as a 1.5 ml microcentrifuge tube) for subsequent steps.

For scanning electron microscopy (SEM), specimens may be as large as necessary, keeping in mind limitations of microscope chamber size and specimen exchange.

Primary fixation is normally done in an aldehyde-based fixative in a buffer: Aldehydes may be glutaraldehyde, paraformaldehyde, or formaldehyde, or combinations of these.

(NOTE: formaldehyde without glutaraldehyde should be restricted to situations where preservation of fine detail is not necessary. This is considered primarily to be a histological fixative, not an electron microscopy fixative.)

A general-use primary fixative would be 2% glutaraldehyde/2% paraformaldehyde in 0.1M cacodylate or other suitable buffer (PBS, HEPES, PIPES).

Note: If precipitation is visible in final sections, we recommend changing to a buffer containing 2-Mercaptoethanol (0.1M Na Cacodylate, 0.13M Sucrose, 0.01M 2-Mercaptoethanol---see Common Buffers and Fixatives page). This can prevent osmium-based pepper in samples.

Secondary fixation of specimens takes place in 1% or 2% osmium tetroxide either aqueous or in the same buffer as used in the primary fixative. ***Osmium fumes are toxic and can fix exposed skin and eyes! Perform osmium procedures in a fume hood and use gloves!***

Tertiary fixation, or *enbloc staining*, if desired, is normally in 1-2% aqueous uranyl acetate. This serves both as fixative and stain and can markedly enhance membranes.

Specimen Preparation for TEM

Processing step times are variable, but the following times are average. In general, for plant materials or other difficult-to-infiltrate specimens, times should be extended, for cells in suspension or on cover slips they may be reduced---refer to published studies for examples.

- 1) Immerse samples in primary fixative for 1-2 hours on rocker (room temperature) or several hours at 4 degrees C.* (Samples may normally be stored in fixative if processing cannot be completed immediately.)
 - 2) Replace fixative with buffer and place on rocker. Repeat three times for 15 minutes each wash. (Discard all used reagents in an appropriately waste container).*
 - 3) Replace buffer with secondary fixative for 1-2 hours on rocker at room temperature, preferably in a fume hood when using osmium.
 - 4) Wash in ultrapure distilled water, 3 x 15 minutes each on rocker.
 - 5) Tertiary fixation for 1-2 hours, if desired.
 - 6) Wash in distilled water, 3 x 15 minutes each.
 - 7) Dehydrate in graded acetone series: 30 minutes each in 25%, 50%, 70%*, and 95%. Dehydrate in changes of 100% for 30 min., 30 min., and 1 hour. (The 100% acetone for the last steps should be freshly opened or stored with molecular sieves.)
- Note: Make sure processing container is compatible with acetone use. Use of acetone permits skipping the use of carcinogenic propylene oxide as a transitional solvent into the resin infiltration steps. Ethanol may also be used for dehydration, but is not fully compatible with some resins, requiring a transitional solvent. Acetone may be used as a transitional solvent after ethanol, if you prefer not to use it for the entire dehydration process.*
- 8) Begin infiltration with 1 part resin: 2 parts acetone for 2 hours.
 - 9) Continue infiltration with 1 part resin: 1 part acetone for 2 hours.
 - 10) Infiltrate with 2 parts resin:1 part acetone for 2 hours.*
 - 11) Pure resin overnight or for 8 hours.*
 - 12) Pure resin for 4-8 hours.*
 - 13) Put fresh, pure resin in embedding capsules or molds and add specimens and labels (if using them).
 - 15) Polymerize in 50-60 degree C oven for one to two days. Consult manufacturer's instructions for each different resin.

16) Make ultra-thin sections, and place on grids.

17) Stain grids in uranyl acetate, followed by distilled water washes and lead citrate staining (times vary), then do final washes in distilled water.

18) View in TEM.

* Indicates possible overnight stopping point.

Specimen Preparation For Immunolabeling in TEM

Follow the above procedure, except:

Step 1: Use a fixative with less gluteraldehyde. An acceptable fixative could be 0.5% gluteraldehyde/4% paraformaldehyde in 0.1M cacodylate buffer.

Proceed directly to Step 6 above, do not perform secondary or tertiary fixation.

Steps 7-15: Use a resin designed to retain maximum antigenicity in tissues, such as LR White, Lowicryl, or other suitable resin. Perform the dehydration steps according to instructions with the resin, however we recommend always dehydrating at least through 100% ethanol whenever possible, even if the manufacturer says it's not necessary. Polymerize resins at manufacturers' recommended times and temperatures.

Step 16: It is recommended to use nickel or gold, rather than copper, grids for immunolabeling. Due to the more fragile nature of most resins used in immunolabeling, you may wish to use a smaller mesh grid for greater support of your sections, as well as a support film on the grid, such as carbon or carbon/formvar. This will help prevent sections from tearing and disintegrating during labeling, staining, and viewing.

Step 17: Perform contrast (UA and/or Pb) staining procedures AFTER immunolabeling your grids (see general instructions for immunolabeling).

Specimen Preparation for SEM

Perform steps 1-7 for TEM prep, then continue as follows:

8) Critical point dry specimens (see EM Core staff).

9) Mount on suitable SEM stubs with appropriate glue, tape, or adhesive disk.

10) Sputter coat with Au, Au/Pd, Cr, or Pt.

11) View in SEM.