Practical Methods in High-Pressure Freezing, Freeze-Substitution, Embedding and Immunocytochemistry for Electron Microscopy

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Practical Methods in High Pressure Rapid Freezing,
Freeze Substitution, Embedding and Immunocytochemistry
for Electron Microscopy

This manual is a technical guide to methods in high pressure freezing and subsequent freeze-substitution fixation and embedding for high resolution electron microscopy and EM-immunocytochemistry. It contains a number of methodologies which we have found useful in various combinations to provide acceptable-to-superior preservation of a variety of biological samples. Many of these methodologies are relatively new and still require experimentation for certain biological systems; however, this manual will provide some critical ground work for future investigations.

Introduction

Fixation of biological samples for preservation of fine structure has been a goal of structural biologists since the beginnings of electron microscopy. The goal has always been to preserve samples as close to their native state as possible with resolution that could be achieved by the technology of their time. As microscopic technology and computer capabilities have grown so has interest in the rapid fixation of biological samples. Electron microscopes with high tilt stages have made it possible to reconstruct tomographic slices through volumes of biological material with 5nm or better resolution. Thin sections have been used to reconstruct structures or systems over several microns through a sample and immunocytochemistry techniques have allowed for the high resolution localization of gene products in various systems of interest. These advances have pushed (if not exhausted) the limits of chemical fixation as a means of generating reliable data for high resolution microscopy, resulting in an increased interest in ultra rapid freezing (cryofixation) as a means of immobilizing biological fine structure.

Cryofixation has two distinct advantages over chemical fixation: a much faster rate of fixation and a simultaneous stabilization of all cellular components. The low-temperature substitution of dehydrating agents and fixatives (freeze-substitution) into rapidly-frozen samples allows for the crosslinking of cellular components and the removal of water at temperatures low enough to avoid the damaging effects of ambient-temperature dehydration. Successful cryofixation followed by freeze-substitution, shows superior preservation of fine structure over that yielded by chemical fixation techniques. In addition, the advantages of low-temperature embedding for immunolabeling has greatly contributed to our ability to preserve and accurately localize antigens at high resolution.

Chapter 1 - Rapid Freezing by High Pressure

A) Theory of Freezing

Ultrarapid freezing is the freezing of a biological sample at a rate so rapid that the ice is vitreous or that the ice crystals that form are smaller than can be detected in the electron microscope no larger than 10-15nm. The four most common methods of cryofixation are 1) plunge freezing, 2) propane jet freezing, 3) cold metal block freezing and 4) high-pressure freezing. Successful freezing with the first three of these methods is limited to only very thin samples such as a monolayer of cells. Only high pressure freezing enables samples with a thickness of up to 0.6mm to be well frozen. A detailed and comprehensive review of the above methods can be found in Gilkey and Staehelin, 1986. The necessary freezing rates of -10,000°C/sec can only be achieved for very thin layers (<20μm) when samples are frozen at atmospheric pressure. Thicker samples can only be well frozen if these critical freezing rates can be reduced by changing the physical properties of water in three ways: 1) lowering its freezing point, 2) reducing the rate of ice crystal nucleation, and 3) slowing the growth of ice crystals. Permeating cryoprotectants, such as glycerol or DMSO can be used to inhibit ice crystal formation, but at the risk of introducing structural changes. Application of high pressure (~2,050 bar) immediately prior to freezing a sample can change the freezing behavior of water enough to cryopreserve thick biological specimens with minimal ice crystal formation (Dahl and Staehelin, 1989). (For a detailed treatment of the physical aspects of freezing, see Gilkey and Staehelin, 1986, or Moor, 1987). The practical methods discussed in this manual will refer only to high pressure freezing.

B) Preparing for Freezing in the High Pressure Freezer (HPF)

The main goal of rapidly freezing samples is to preserve fine structure of cells in their native and physiologically active state. It is therefore important to minimally perturb the sample and to maintain normal growth conditions right up to the initiation of freezing. There are a number of ways to help achieve these goals:

1) Plan ahead. Always have waterbaths or incubators close at hand if needed. If necessary, provide warmed stages for dissection or LM examination of samples. Keep tools for loading close at hand. Maintain pH of solutions used.

2) Practice loading material into freeze specimen holders (planchettes) before attempting to freeze real samples. Try growing samples under various conditions to ease their transfer into holders. If using cryoprotectants (see below), experiment with conditions that allow cells to develop normally after treatment with cryoprotecting compounds.

C) Specimen Holders

There are a variety of sample holders (hats, planchettes) designed for use in the HPF, and

the choice of holder varies with sample to be frozen. The key point is to use a holder whose internal dimensions are large enough to accommodate the sample without mechanically damaging it, yet small enough to minimize extra space (and thus unnecessary volume from which heat must also be extracted). All of the enclosed air spaces should be filled with sample or fluid, since air pockets or small bubbles usually result in sample damage or loss. Different manufacturers provide various designs along a similar theme, namely a top "hat" and bottom "hat" with a variety of inner-well dimensions which can be used interchangeably to provide an appropriate inner cavity for the sample. Practical points of preference and use follow.

1) Specimen Holder Choices

a) Copper or aluminum - These usually come with one type "welled" and the other smooth, so that two welled hats can be used to create a larger cavity, while a welled and flat decreases the cavity dimension by half. These hats are "disposable" as they may become bent or corroded during freeze substitution. (Vender: Technotrade International)

b) Custom-made brass hats - These interlocking hats (Craig, 1987) are held together tightly during pressurization and freezing, resulting in less specimen loss and reduced fracturing of the sample. While these hats are expensive to manufacture, they can be reused after cleaning with sonication. (Vender: Swiss Precision, Inc.)

c) Gold holders - These holders may vary in dimension but are only used if it is necessary for samples to grow in them for long periods of time, as other types of metals corrode and become toxic to the living cell.

d) Others - Custom holders may be designed to fit a particular specimen provided they fit the outer diameter of the freezing rod tip. For example, we have enlarged the inner cavity of the brass hats to accommodate an early *Xenopus* embryo, and while this dimension pushes the limits of freezing depth, some parts of these embryos exhibit good freezing.

D) Additives to Cells and Tissues

While the size and geometry of the specimen holder should be optimized to provide the best freezing rate possible, the medium surrounding the sample is probably even more critical to the quality of freezing obtained. The entire volume of the inner cavity of the specimen holder must be filled. Air bubbles within the holder act as insulators and collapse during pressurization that can deform a sample milliseconds before freezing (Dahl, Staehelin 1989). Freezing success varies between sample type, due to differences in water content of different organelles, or as a result of naturally occurring cryoprotectants within different samples. For most samples, the best choice of "filler" for the specimen is one which has some cryoprotection ability. Also consider the potential effects of the chosen cryoprotectant on downstream processing steps as discussed below.

Cryoprotectants

Cryoprotectants improve the quality of freezing directly by suppressing the formation of extracellular ice crystals and indirectly by reducing the amount of heat released by the crystallization process, thereby increasing the overall rate of cooling of the sample (Dahl, Staehelin, 1989). Since the premise of these procedures is to preserve samples without perturbing their natural process, a number of cryoprotectants can be used to accomplish this goal.

1) Non-penetrating and hydrophobic substances with low osmotic activity (aka. extracellular)
   a) dextran (15-25%, MW 39,000)
   b) polyvinylpyrolidone (15%)
   c) serum albumin (10-20%)
   d) Ficol (5-15%, MW 70,000)
   5) low-melt agarose (0.5-2.0%)
   6) cold water fish gelatin (50-100%)
   7) 1-hexadecene
   *Note: Hexadecene is immissible with water and can therefore be considered inert. It is useful for filling specimen holders while minimizing the volume of aqueous material to be frozen.

2) Penetrating (in some tissues) (aka. intracellular)
   a) sucrose (150mM)
   b) methanol (10%) ± paste of yeast for filler
   *Note: The penetration of these depends upon the tissue. Thick cell walls are likely unperturbed by the brief addition of sucrose while mammalian tissue culture cells with delicate dynamic processes may be affected. *Drosophila* embryos develop normally after 10 minutes in 10% MeOH, but cultured mammalian cells in monolayers do not.

E) Sample Loading - Practical Suggestions

The following are examples of successful methods for given samples. These procedures may be combined or altered as fits the need of the system. For the following, a dissecting microscope set-up in close proximity to the freezer is invaluable for sample loading.

1) Suspensions of cells

   a) Plants (Samuels, Giddings, Staehelin, 1995, Tobacco BY-2)
   - Grow cells accordingly
   - Suspend cells in 25% dextran (39,000 MW)
   - Concentrate on 30μm nylon mesh
   - Transfer cell slurry w/micropipette or dissecting needle to brass sample hat for freezing and storage in LN_2

b) Mammalian cells (Schlegel, Giddings, et al., 1996, HeLa)
-Dislodge cells from culture dish with rubber spatula following brief trypsin treatment
-Centrifuge 3 min at 240 x g
- Suspend cells in 0.15M sucrose culture medium
- Centrifuge 3 min at 240 x g
- Discard supernatant and vortex pellet in minimal volume to create thick slurry
- Aliquot slurry by micropipette to brass hats for freezing and storage in LN_2

c) Yeast (*S. pombe & S. cerevisiae*)
- Grow cells to early log phase
- Collect cells on 0.45µm millipore filter through vacuum filtration, taking care not to dry the filtered cells
- Scrape cells from filter with a flattened spatula
- Transfer a slurry of cells with a sharpened toothpick to a brass freezer hat for freezing and storage
*Note: A "slurry" is a consistency like apple sauce. If cells are too wet, they won't freeze well. If they are too dry, they won't embed well.

2) Monolayers
These methods are useful for correlative LM/EM studies and for cell selection and reorientation prior to sectioning.

a) Mammalian Tissue Culture Cells
- Cut small rectangles (or appropriate dimension to fit hat) of thermanox (Nunc) coverslip. Sterilize about 20 chips of thermanox plastic in each of 3 to 5 - 35mm dishes. Flood with media (these will float, so push down with sterile forceps). Plate cells to dishes and monitor growth (2-3 days).
- When cells have grown to desired confluence, examine on warmed light microscope stage with water immersion lens prior to freezing (scope set-up by freezer).
- Add warmed culture medium supplemented with 15% Ficol (MW 70,000) to the well of a Cu or Al freezer hat with a micropipette or pulled glass pasteur pipette.
- Transfer plastic chip with cells quickly to this well. Freeze and store in LN_2.

b) Fungi
- Embed freezer hats (any type) in the superficial layer of agar or other substrate on which the sample grows.
- Seed sample close to hats so that their processes grow over the hat-agar substrate. Allow for optimal growth.
- Score the outer edge of the hat with a fine tungsten needle and carefully remove the hat. Fill the top hat with low melt agar (0.5-2%), cap and freeze for LN_2 storage.

3) Tissues

If it is desirable to recover these samples intact rather than as two fractured halves, coat the top hat with a freshly prepared solution of vegetable lecithin (100mg/ml in chloroform). This lecithin coating in the top hat will act as a non-stick surface so that the bottom hat retains the entire sample.

a) Plant root-ups (Staehelin, Giddings 1990)
-Transfer seedlings to a drop of 15-25% dextran (MW 38,800)
- Excise 1-2mm tips from three to four roots with a razor blade
- Transfer root tips and dextran with capillary pipette to Cu or brass freezer hats
Freeze and store in LN_2.
-0.15M sucrose 0.5% agarose can be substituted for dextran (dextran may be difficult to embed in Lowicryl)

b) Animal tissue (Keene,P.U.)
It is important, yet difficult to dissect a small (0.5mm) slice of tissue from an organ without mechanical damage to that small slice. A punch-biopsy tool, or homemade apparatus of two microscope slides onto which are glued two thin razor blades can be used as a cleaver.
- Cut a small sliver of tissue from the sample
- Transfer to appropriate-sized freezer hat containing either 15% dextran or 15% 1-hexadecene. Freeze and store in LN_2.

4) Embryos

a) *Drosophila melanogaster* (McDonald, Morphew 1993)
- Harvest embryos from an agar plate using a stream of water and small paintbrush onto 100μm nitex screen.
- Remove chorion by washing in 50% bleach for 2 min and rinsing in water 5 min.
- Float filter containing embryos on a small petri dish containing tap water.
- Make a paste of Baker's yeast in 10% methanol to the consistency of pudding and apply a dot of this with a toothpick to both top and bottom of brass freezer hats. Small air bubbles will begin to form in this paste over time, so fresh paste should be made when this occurs as these air bubbles will hamper the freezing.
- Use a fine paintbrush to transfer about 20 embryos to the paste-containing hats using filter paper to blot excess water from the brush. Tease the embryos down into the paste within the holders. Freeze and store in LN_2.

b) Sea Urchin (*Strongylocentrotus purpuratus*) (McDonald, Morphew 1993)
- Collect eggs from animals by KCL injection and allow them to develop in calcium-free sea water.
- Concentrate by hand centrifugation in an eppendorf tube and remove as much

supernatant as possible without drying embryos.
- Resuspend in a small volume of either 1-hexadecene, 15% dextran, or fish gelatin. - Transfer to brass or aluminum hats with micropipette for freezing and storage.

c) *Caenorhabditis elegans*
- Transfer early embryos with a micropipette onto an agar growth plate in groups of 5-10. Allow to develop to desired cell stage.
- Excise small squares containing embryo groups with scalpel and transfer to brass freezing hat. Cover this agar square with enough volume of low melt agar as needed to fill the bottom well. Fill top hat with either 0.15M sucrose or 15% Ficol (MW 70,000) both dissolved in embryo saline. Freeze and store in LN_2.

Chapter 2 - Freeze Substitution

Specimens that have been cryofixed by high pressure freezing may be processed in four ways: 1) direct viewing of frozen specimens on a microscope cryostage, (Dubochet et al., 1987), 2) sections cut of frozen material in a cryoultramicrotome (Michel et al., 1992), 3) freeze fracture (Craig and Staehelin, 1988), Moor et al., 1980), and 4) freeze-substitution. The first two methods are technically demanding, but much useful data has resulted in these very difficult studies of frozen hydrated tissues. Freeze fracture is commonly used to examine membrane systems in frozen samples with great success. However, the focus of this chapter is on freeze-substitution as a means of examining cryofixed material by conventional microtomy and microscopy.

Freeze-substitution is the process of dissolution of ice in a frozen specimen by an organic solvent at low temperature and usually takes place in the presence of a secondary fixative (Steinbrecht and Müller, 1987). Freeze-substitution is usually carried out at temperatures below which secondary ice crystals may grow (i.e., below -70°C). Once substitution is complete, samples may be warmed-up without recrystalization, as water is now absent from the sample. The main advantages of this method are: 1) dehydration occurs at low temperature, thereby greatly reducing the occurrence of ultrastructural changes often seen from room temperature dehydrations; 2) fixatives are uniformly distributed throughout the sample prior to warming, which is when their crosslinking activity begins, and 3) sample intended for immunocytochemistry may be infiltrated with resin and polymerized at low temperature, thereby reducing the damaging effects that ambient-temperature organic solvents and heat polymerization may have on some epitopes.

A) Equipment for Freeze-Substitution

Some fine commercial devices for this method are available, while many homemade systems work equally well. The basic design is a chamber which will maintain a sample in solution at -90°C for 2-3 days and can then be warmed-up slowly to a variety of graduated temperature points. Some examples follow.

1) Commercial Models (Leica)
   These units are cooled by liquid nitrogen and are convenient as they may be programmed for warming. They are also equipped with a system for precooled solution exchanges for resin infiltration and, in addition, are fitted with an Ultraviolet lamp for low-temperature polymerization of resins used in cytochemistry.

2) Homemade LN_2-cooled Basket
   This type of system requires some machining and moderate expense. The samples are held in a metal block into which holes have been drilled to accommodate 2ml cryotubes (in which samples will reside). This metal block is

wrapped with heater wire and hangs in a LN_2 dewar. The level of liquid coolant (LN_2) is kept below the hanging block, so that N_2 gas is surrounding and cooling the block. The heater wire is connected to a temperature controller, and one cryovial contains a thermocouple to maintain the block at a set temperature (-80°C). This unit can either warm-up passively when LN_2 runs out, or can be reset for warmer temperatures for low temperature embedding.

3) Homemade Box

This simple yet effective set-up consists of a styrofoam box and a metal block with holes drilled to accommodate a 2ml cryotube for samples. The box is packed with a mixture of dry ice/acetone to maintain temperatures about -80°C. When the user allows the dry ice to exhaust, the block will warm slowly to a desired temperature, after which samples are transferred to a chest freezer (-20°C) or ice (0°C) for the appropriate embedding procedure. Other intermediate temperatures can be achieved if necessary by mixtures of MeOH and dry ice.

B) Recipes for Substitution

1) Choice of Solvents:
A variety of solvents may be used for substitution. In our hands, acetone continues to provide the most satisfactory results with well preserved fine structure and minimal extraction, and most recipes that follow suggest its use. However, methanol has the advantage that it can substitute specimens in the presence of substantial amounts of water and substitution is faster at low temperature in methanol than in acetone (For a review of additional substitution solvents, see Robards and Sleytr, 1985 or Steinbrecht and Müller, 1987).

2) Recipes for Ultrastructure

a) Common generic subfluid and suggested schedule. (Other variations below may be added as appropriate.)
<1% OsO_4 in Acetone>
-prepare stock solutions of 4% OsO_4 (from crystals) in anhydrous acetone. Aliquot to a number of 2ml cryovials and store for further use. Osmium in acetone will react more quickly than when dissolved in H_20, so these stocks should be made and frozen without delay. OsO_4 is very toxic and reactive. Always wear gloves and work in a fume hood! -remove a 4% OsO_4 stock from storage, loosen top of vial before thawing is complete to avoid "pop" from trapped gas.
-dilute with anhydrous acetone to make 1% OsO_4.
-realiquot to desired number of 2ml cryovials.
-refreeze these vials in LN_2.

-transfer frozen samples into frozen fixative under LN_2. Always handle frozen samples with precooled instruments. (Interlocking-style hats should be separated top-from-bottom under LN_2 before this step. Other types of holders will open by themselves during substitution).
-transfer frozen vials with samples to precooled (-90°C) substitution vessel.
-as the sample vials warm from -196°C to -90°C, the sub-solution will thaw and samples will sink to the bottom. A brief daily agitation of the vials is useful, provided they are not allowed to warm above -80°C for 3 days.
-substitute for 3 days at -90°C.
-warm samples to room temperature over 18 hrs to 2 days.
rinse samples in anhydrous acetone for further resin infiltration.

b) Variation 1 - <0.1% Uranyl Acetate + 1% OsO_4 + Acetone>
The addition of uranium helps with membrane contrast
-make 1% stock solution of UAc in acetone.
-add appropriate amount to final sub-solution and proceed as above.

c) Variation 2 (Ding, et al 1991)

<0.1% Tannic Acid + Acetone> -90°C- 1 day
¬
<rinse acetone X 3 > -90°C
¬
<1% OsO_4 _0.1% UAc + acetone> -90°C 2 days
¬
warm to RT, rinse acetone 3X

This protocol greatly improves filament preservation, but may provide too much contrast for some membrane systems. It requires solution exchanges at -90°C which can be challenging and tedious, but the results are often remarkable.

d) Variation 3 - ,0.1% Tannic Acid + 0.5% Glutaraldehyde + Acetone> -90°C 1 day
¬
<rinse acetone 3X> -90°C
¬
<1%OsO_4 _0.1% UAc + acetone> -90°C 2 days
¬
warm to RT, rinse acetone X3

This protocol is similar to variation 2, except that glutaraldehyde has been

added in the first step. This has improved the fixation of the thin monolayers of cultured cells where some extraction of the cytoplasm was noted with OsO_4 only fixes.

3) Recipes for Immunocytochemistry

Fixation for immunocytochemistry can be a tricky business because of the vast difference of epitopes and the strength and specificity of the antibodies meant to label them. In most cases, the crosslinking of fixatives must be weak enough to render the epitope available to an antibody, yet strong enough to preserve fine structure to which antibodies may localize at high resolution. Some epitopes are so sensitive that no chemical, however dilute, may be used, while others show vigorous label after the strongest of osmium exposure. If possible, these limits should first be tested by light microscope techniques as these EM procedures are lengthy and labor-intensive.

Some suggestions for immunocytochemistry-freeze substitution follow. The transfer of samples to fix solutions follow the protocol in the previous section.

a) 0.1%-0.5% glutaraldehyde in acetone.
Glutaraldehyde is available as an anhydrous solution from EM vendors, but we have diluted 70% aqueous stocks with equal success.
-substitute at -90°C for 3 days.
-warm to -50°C or -35°C for embedding in Lowicryl HM20 or Lowicryl K4M respectively.

or

-warm to 0°C for embedding in LR White.
-rinse 3X in acetone at the appropriate temperature for further resin infiltration.

b) 2% - 4% paraformaldehyde $\pm$0.1% glutaraldehyde.
Paraformaldehyde powder is not soluble in acetone so:
-make 20% solution of paraformaldehyde in anhydrous MeOH.
-cover, gently warm and mix on heated stir plate until condensation begins around upper edges of the beaker.
-add ~1/4 to 1/2 pellet of NaOH until the solution clears.
-dilute to 2%-4% with acetone; proceed as above.

c) Acetone only

Acetone alone can yield results of fair quality for very sensitive antigens, especially when combined with embedding in Lowicryl HM20 at -50°C.

d) 0.01% - 0.1% OsO_4

Some vigorous epitopes and antibodies continue to work well after OsO_4 fixation, especially plant polysaccharides. In this fortunate case, grids with sections of osmicated tissue should be floated on a drop of saturated sodium

metaperiodate for 10 minutes to remove bound osmium. Tissue which significantly blacken from osmium fixation are not suitable for UV polymerized resins, as these blackened areas will not crosslink.

Chapter 3 - Embedding and Sectioning

A) Embedding and Sectioning for Ultrastructure

Infiltration, embedding, and sectioning of samples that have been cryofixed and freeze-substituted follow general guidelines for that of conventionally fixed specimens; however, some adjustments should be made. The cryofixed samples have a more dense cytoplasm and show less extraction from dehydration, so infiltration may require extended times. In addition, many samples which benefit from cryofixation are those which are difficult-to-fix by conventional means, due to their thick cell wall and hard-to-penetrate egg shells. Since these samples retain their walls and shells in these protocols, care must be taken to slowly and thoroughly infuse the samples with resin through many graduated steps. Suggestions follow.

1) Resin - both Epon-Araldite (or other Epon substitutes) and Spurr's resin are commonly used with success. Epon is more viscous but sections well and has superior post-staining results. Spurr's resin is less viscous, but is more difficult to section and post-staining is less dramatic. Other formations work well and experience and preference are the deciding factor. Accelerator should not be added until the final change in resin.

2) Plant tissues, yeast embryos and other thick-walled organisms (all on rocking platform);
-10% resin: 90% acetone - overnight
-25%, 50%, 75% resin, -4 hrs each or overnight
-100% resin overnight
-100% resin + accelerator - 2 hrs
-transfer samples to BEEM capsules, or flat-ended molds for polymerization at 60°C.

3) Cell monolayers or other easily infiltrated tissue
-30%, 50%, 75%, 100% resin - 2 hrs each on rocking platform
-100% rein and accelerator - 2 hrs
-transfer samples to appropriate molds for polymerization
*For cells grown on plastic chips (namely resulting from sec I-E-2 of this manual) and for embryos or others samples which require selection it is useful to review polymerized samples in the LM for cell selection and reorientation. Final embedding for these samples is:
-generously spray two microscope slides with spray lubricant release agent (Miller-Stevenson); allow 5 minutes to dry and wipe clean with Kimwipe
-add thin strip(s) of parafilm along the short dimensions of one slide to act as spacers to avoid sample compression (this is not necessary with thin cell monolayers)
-spread a strip of resin plus sample along the length of one slide (volume of this strip should approximately equal that of two standard toothpicks). Place the second slide on top with opposing overlap of ~2cm.
-polymerize at 60°C on a platform of two applicator sticks to prevent trapped resin from wicking over slide sides.

These two slides can be easily separated after polymerization and the resulting wafer of embedded sample can be viewed and scored in the LM.

B) Embedding for Immunocytochemistry

Section II-B-3 previously suggested that the choice of substitution solutions for immunocytochemistry varies depending upon the antigen to be investigated and the antibody available. This is equally true for the embedding medium, and temperatures of infiltration, and polymerization. Unlike fixation variables, these parameters cannot be tested in the LM, so one may choose to start with the technically less demanding choices and progress as needed. Those fortunate enough to retain label after osmium fixation and epoxy embedding are few, but it's worthwhile to give this a try before embarking on the acrylic (LR White, LR Gold) and methacrylate (Lowicryl K4M, HM20) resins whose use is more demanding. These choices are listed in order of their ease in implementation.

1) Epoxy resin with OsO_4 fixation

This combination is successful for polysaccharide staining of plant tissues and where other vigorous antibody/antigen combinations exist. Treat these samples as in sections IIIA 1-3. Sections on grids should be bathed in a saturated solution of sodium metaperiodate for 10 minutes prior to immunolabeling.

2) LR White

LR White is an acrylic resin and is useful for many application in immunocytochemistry. It is easy to work with as it is purchased as a complete mixture with only the addition of accelerator lacking and it is less toxic than its methacrylate counterparts. It is also of low viscosity, so it infiltrates tissues well. The sectioning properties of LR White are good despite its hydrophilic properties and its brittle nature when trimming. The disadvantages of LR White are that it is usable from 0°C to room temperature and requires heat (45°C) for polymerization, so heat-sensitive epitopes may not survive its use. In addition, LR White polymerization is sensitive to the presence of 0_2, so flat-embedded samples are impractical, though not impossible. Further, LR White seems to result in some more extraction and shrinkage (as seen by "halos" around nuclei) than other possible embedments.

Suggestions for LR White use following freeze substitution:

-maintain samples at 0°C (on ice) after freeze-substitution and throughout infiltration.

-exchange graded series of resin:solvent times depending upon tissue used. Add accelerator at the final step only.

-1:2, 1:1, 2:1 (2 hrs each) 0°C

-full strength overnight 0-4°C

-change 2X full strength (this is important if acetone is the solvent of choice).

-mix accelerator and resin according to manufacturer's directions and exchange 1 hr - 0°C.
-transfer sample and resin to gelatin capsules. Fill capsules to the brim with resin before capping.
-polymerize overnight at 45°C in a vacuum oven.

3) Lowicryl (K4M or HM20)

The Lowicryl resins are perhaps the two most popular embedments for immunocytochemistry following cryofixation, likely because of their use at low temperature. This advantage allows for good preservation of ultrastructure (as minimal warming in organic solvent occurs) and for retention of antigenicity (as resin is polymerized by UV light at low temperature). These methacrylate resins bond with cellular components in different ways than their epoxy counterparts and they tend to "fracture" rather than section cleanly in the microtome, making sites of antigen staining available at the surface of their sections (Ref, Kellenberger 1987). These advantages usually outweigh their disadvantages when epoxys and acrylics are inadequate or inappropriate for a given system. Among the disadvantages of these methods are the technical efforts required to maintain samples at low temperatures while infiltrating, embedding and polymerizing them. In addition, and of import, is the toxic nature of these volatile methacrylates. Adequate fume hoods, appropriate gloves and protective glasses should always be used when dealing with these resins!

Lowicryl resin is available in four types and a thorough discussion of each and its uses is reviewed in Kellenberger and Carleman, 19 __. This author's experience lies with only K4M and HM20, so those are presented here.

Lowicryl K4M is a polar, hydrophilic resin used at -35°C and above. This resin is of low viscosity and reasonable temperature ranges for its use may be achieved in a chest-type freezer at -20°C. Polymerization is then possible in a styrofoam box placed within that freezer. Flat-embedding in the resin is possible in 0_2 free environments. This resin is not friendly at the knives'edge in a microtome, and its hydrophic properties make it difficult to collect long ribbons of thin section.

Lowicryl HM20 is a nonpolar, hydrophobic resin useful at -50°C and above. This is a very low viscosity resin, which makes it useful at low temperatures where infiltration exchanges are difficult due to the slow movement of molecules at these temperatures. The use of this resin requires a chilled vessel for infiltration and embedding, (see suggestions below), and flat embedding is also possible with this resin. Lowicryl HM20 sections as easily as any epoxy resin, producing long, consistent ribbons of serial sections.

a) Lowicryl K4M or HM20

-Maintain temperature for freeze-sub, solvent rinses, infiltration and embedding (-

20 to -35°C for K4M) and (-50°C for HM20). Proceed in a similar fashion for both resins, extending or abbreviating infiltration times as appropriate for a given tissues. The following is a suggested schedule for yeast.
-Prepare fresh resin for each experiment and mix the components according to the manufacturer's suggestion.
-Infiltrate samples with 30%, 50%, and 70% resin in acetone (MeOH or ETOH if used as substitution solvent) for 4 hrs each.
-Exchange to 100% resin overnight.
-Replace with fresh 100% resin for 2 hrs.
-Transfer samples to precooled BEEM capsules or to flat-embedding substrates for UV polymerization.

b) Suggestions for equipment: When commercial models are not available, or their use is inappropriate for an application, reasonable homemade rigs can be designed. Since it is important to avoid inhaling the vapors, a shallow styrofoam box which fits in a fume hood filled with dry ice can act as a cool chamber. A metal block with holes drilled to accommodate 2 ml cryovials is a useful holder, and temperatures ranging from -20 to -70°C can be achieved by the addition of various volumes of MeOH or ETOH. A digital thermometer with a thin thermocouple wire is a useful way to monitor the temperature within a cryovial.
c) UV polymerization

Both Lowicryl K4M and HM20 are cured by UV polymerization of 360nm wavelength at temperatures between -20°C and -50°C. Samples in BEEM or gelatin capsules should be immersed in a cooled fluid (MeOH, ETOH) so that the heat generated during polymerization may be dissipated. Cure samples for 24 to 48 hrs at low temperature under indirect UV irradiation, and an additional 48 hrs at room temperature.

When commercial devices are unavailable, a variety of substitutes are useful. A simple styrofoam box lined with aluminum foil, mounted with a hand-held UV lamp and placed in a chest freezer a -20°C is simple, yet effective. A more elaborate set-up requires a large insulated metal box partially filled with dry ice chips. A metal cooking bowl wrapped with a heater and filled with solvent (and containing a sample rack) is connected to an external temperature controller. Two 360nm UV bulbs mounted 40cm above the sample rack are shielded by a metal plate to prevent direct bombardment of the sample by strong UV rays.

d) Flat embedding in Lowicryl

Many sample types are most useful if they can be re-evaluated and reoriented in the light microscope prior to sectioning. This is a possibility with Lowicryl, provided the chamber for polymerization is filled with gaseous CO_2 or N_2. Suggestions for samples appropriate for this method (embryos require stage

selection, cultured cells grown on plastic chips discussed previously, or others) follow.

-spray one side of each of two thermanox coverslips (22x22mm) with Spray Lubricant Release Agent (Miller-Stevenson), dry 5 minutes and wipe clean.

-cut a center square from a third thermanox slip with a scalpel and obtain a "picture-frame" (~3mm x 3mm) of coverslip.

-glue this frame (with "Super-glue" or other methacrylate-based glue) to one prepared coverslip. This frame will act as both a spacer for samples and as a "dam" to contain resin.

-place coverslips in aluminum weigh dishes, add a few chips of dry ice to the dish to prevent water condensation when chilling, cover with another dish, and precool to embedment temperature.

-pipette sample and resin onto the framed coverslip, taking care not to overload the slip.

-invert the second coverslip (lubricated side down) on top of the framed slip and sample to form a sample sandwich.

-float the weighing dish in cooled solvent under UV irradiation to polymerize.

-this sandwich is separated easily with forceps after curing, and samples may be viewed, scored in the LM, cut to remount and sectioned.

Chapter 5 - Immunocytochemistry

Immunolabeling is the final step in the labor-intensive procedure following cryofixation. This investment of technical time makes it important to characterize antibodies thoroughly by biochemistry and light microscopy if possible, before proceeding.

A) Sectioning Suggestions Prior to Immunolabeling

-Collect sections on nickel grids, preferably formvar-coated slot grids, so that label patterns are not obscured by grid bars. It may be useful to evaporate a thin coat of carbon onto the grids prior to sectioning to avoid section wrinkling throughout the procedure.
-Some immuno-resins are hydrophilic and water in the knife boat persists in wetting the sample block face. This problem is alleviated by applying a bit of saliva to the knife edge, and by pulling the boat water level down as far as possible.
-If ribbons of serial thin sections are difficult, a dilute solution of Weldwood cement in xylene applied to the top or bottom surface of the block may help.
-The nature of immuno-resins often results in the sample falling out or separating from the resin during sectioning. It is useful when trimming blocks to always have a frame of resin surrounding the tissue to "trap" sample in a section when cut.

B) Immunostaining

1) Set-up

As with all steps in sample preparation for electron microscopy, pristine conditions are a must. While it is not necessary for buffers to be sterile, beakers, forceps, and all tools used should be clean and free of oils or soaps. Grids may be handled in a few ways for immunostaining:
a) A humid chamber such as a microscope slide box or petri dished humidified with moist paper towel. Microdroplets of antibody solutions on parafilm will allow a grid to float, and forceps are used for transfer through various rinses.
b) A humidified plastic microdroplet dish (Corning) works well to hold antibodies with floating grids. This may be placed on a magnetic stir plate where low settings allow nickel grids to "spin" upon solutions.
c) A modified grid box or grid staining box works well when many grids and variables are to be handled at once. Small holes drilled through the back and sliding cover will contain grids but allow for bulk washes in 100ml beakers. For antibody incubations, a piece of tape covers the holes to contain ~15µl of solution.

2) Blocking Buffers

Solutions to help prevent nonspecific bending of antibodies follow. The usefulness of any of these (or combination thereof) may be tested in the LM or on western blots. Incubate grids 30 minutes at room temperature before primary antibody

and dilute antibodies in this buffer.

a) 0.8% BSA
   0.1% fish gelatin (Amersham)
   0.01% Tween-80 in phosphate buffered saline (PBS)
b) Nonfat dry mile in PBS
c) 10% normal goat serum
   (combinations of any of the above may be useful)

C) Antibody Applications

Primary antibodies should be tested in a range of dilutions. It is reasonable to use LM dilution as a starting range for EM, but often (though not always) one needs higher protein concentrations for EM studies. A standard dilution for secondary gold conjugates can be found and relied upon. We have found that gold conjugates from BioCell ( ) work well at dilutions of 1:20.

-Incubate blocking buffer (BB) - 30 min, room temperature (RT) on rocking platform.
-Blot (don't rinse)
-Apply 1°antibody, diluted in BB- 2 hrs, RT or overnight at 4°C.
-Rinse 3X w/PBS + 0.1% Tween-80 (PBST)
-Apply 2°gold conjugate - 2 hrs, RT
-Rinse 2X w/PBST, 2X w/PBS
-Post fix in 0.5% glutaraldehyde. This step helps to retain bound gold throughout subsequent post staining steps.

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Appendix 2

Supply and Vendor Information

Brass Freezer Hats (interlocking)

Swiss Precision, Inc.
908 Industrial Ave.
Palo Alto, CA. 94303

Cold Water Fish Gelatin (#RPN-416)

Amersham Life Sciences
2636 S. Clearbrook Dr.
Arlington Heights, IL. 60005

TFE Release Agent Dry Lubricant (#MS-122N/CO2)
Epoxy Remount Glue (#907)

Miller-Stevenson Chemical Co., Inc.
George Washingotn Highway
Danbury, CT. 06810

Thermanox Coverslips (#174934)

Nunc, Inc.
2000 N. Aurora Rd.
Naperville, IL. 60566

General EM Equipment Suppliers:

Electron Microscopy Sciences
321 Morris Road, Box 251
Ft. Washington, PA. 19034
(800) 523-5874

Ted Pella (Pelco International)
P.O. Box 492477
Redding, CA. 96049-2477
(800) 237-3526