MICROSCOPY 101

We appreciate the response to this publication feature - and welcome all contributions. Contributions may be sent to Phil Oshel, our Technical Editor at:

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Three Quick Hints for Working with LR White Resin

The following "hints" are based on my experience using a Ted Pella 3450 microwave tissue processor:

1) *Vent your microwave tissue processor to the outside.* We have noticed that if samples of LR White are not covered during polymerization, the media does seem to sublimate, then recondenses and polymerizes on all surfaces. This is our experience during the polymerization of LR White in a nitrogen rich chamber heated to 60°C. Using the microwave, consider submersing BEEM capsules, sealed with Parafilm under the cap, in water. This will insure that LR white fumes do not enter the microwave during polymerization. Use the temperature limiting probe to limit the temperature of the water to 70°C and polymerize for 60 minutes, or set to 80°C and polymerize for 45 minutes. A 500 mL beaker with recirculated water at 10 to 20°C should also be included in the microwave during polymerization.

2) We use formvar coated Nickel grids. We prefer slot grids, but use *whatever you prefer. You may want to use uncoated nickel grids if you* wish to label both sides for a double labeling protocol.

3) A progressively lower temperature scheme is best for alcohol dehydrations:

Start with 30% EtOH at 0°C for 10 minutes, then
Lower to - 10°C for another 10 minutes
Add 50% EtOH at 10°C, then
Lower to - 20°C
All subsequent steps to 1:3 90% EtOH:LRW should be at - 20°C

We also leave the samples overnight in 100% LR White at - 20°C, then raise the temperature to ambient for another hour or so prior to thermal polymerization. We might be too careful. Work could be done in the cold room (brrrrr!) to obtain - 20°C.

Doug Keene, Shriners Hospital for Children, Portland, Oregon

An Easy Method For Filling Vacuum Desiccators With Clean, Dry Nitrogen.

People seem to like to store various objects (specimens, pole pieces, specimen holders, etc.) in plastic vacuum desiccators under vacuum, and there are two problems that can arise when doing so:

1) The desiccator may have a small leak, and over time come back up to atmospheric pressure, thus exposing the objects to ordinary air - the situation that is trying to be avoided.

2) Under the influence of the vacuum, plasticizers may bleed out of the plastic from which the desiccator is made and find their way onto the stored objects.

The second of these problems can, of course, be avoided by using glass desiccators, but the possibility of the first arising would still exist.

Fortunately, both problems can be minimized quite simply by filling the desiccator to atmospheric pressure with a dry, inert gas, rather than leaving it evacuated. For most purposes, dry nitrogen would be a satisfactory gas to use here, although helium or argon might be preferred for storing some reactive materials. If the gas Is purchased in a high pressure tank, it is necessary to be sure that it is oil-free, otherwise the fill-gas may carry oil vapors onto the stuff you are storing inside the desiccator,. This would defeat the purpose of the whole operation, a matter discussed on page 64 of my book *Vacuum Methods in Electron Microscopy*

The vapor pressure of water at the temperature of liquid nitrogen is in the realm of 10^{-20} Torr, and the vapor pressure of most oils is even lower, so the gas that is constantly boiling off each container of liquid nitrogen is about as clean and dry as any you can get. As described on page 65 of *Vac. Meth. in EM,*. this dry nitrogen can be collected and used to fill vacuum apparatuses rather simply:

1) Fit a one-hole stopper Into the LN_2 storage flask and connect it to the inlet valve of the vacuum container with noncollapsible, flexible tubing. Ordinary polyethylene tubing works well.

2) Attach a large, collapsible plastic beach ball to a Tee joint in this tubing with a length of soft, surgical-rubber tubing, and make a clean slit in this surgical tubing about 100 mm long with a sharp razor blade or scalpel. Ordinarily this slit will close tightly enough so that the nitrogen gas evolved from the storage container will be directed into the beach ball; however, if the ball becomes full, the slit will serve as a primitive pressure-release valve by opening slightly and allowing the gas to escape so that there is no danger of over-pressurization.

3) When the inlet valve to the evacuated chamber is opened, the nitrogen will flow into the chamber only under the influence of atmospheric pressure acting on the collapsible beach ball, so there is no danger of exceeding atmospheric pressure in the chamber. An ordinary beach ball will hold enough gas to fill most desiccators several times.

If an oil-sealed rotary vane pump is used to evacuate the desiccator, then some care must be exercised to avoid having oil vapors backstream from the pump into the chamber. To avoid this, it is important not to pump the chamber down below the range of viscous flow (i.e., below about 0.1 Torr - see page 29 of *Vac. Meth. in EM). It this is not considered to be a sufficient vacuum to* remove as much atmospheric gas as desired, then the container can be filled with the dry gas, pumped out and filled again a couple of times. Each time the container is pumped out some 90% of the existing gas molecules is removed, so two or three flushings should leave only an insignificant trace of the original atmospheric gases. Alternatively, the problem of the backstreaming of oil during evacuation can be eliminated by using an oil-free rough vacuum pump, such as a carbon vane, diaphragm, or scroll pump. The problem here being that such pumps are not commonly available in most laboratories.

Wilbur C. Bigelow, University of Michigan

A Note On Fixing And Embedding Vertebrate Eyes

I have had experience from mice to whales, and a lot in between. I would recommend Davidson's as a fix. It will keep your retina from going for a walk. Secondly, paraplast is a problem for eyes. A higher melting point wax is needed, without all of the fancy extras. I use Peel Away with a melting point of 56° to 58° C.

The eyes can be processed using another paraffin on the processor, then transferred into Peel Away for the last change in a warming oven for about an hour.

I have processed using both Xylene and Propar, and both worked fine, although you need to adjust the time for Propar.

When I cut, I place my ribbons on to a room temperature distilled water bath, then pick up the sections onto a slide and float them out in a 52° to 54° C water bath.

Place the optic nerve at the label end so it isn't blown out with the heat; it will be evident if the water is too hot, too quickly! I routinely cut at 4 µm,

although larger eyes such as Horse are tough at 4 µm because of the size of the block. The larger eyes will be more successful if cut at 7-8 micrometers in thickness.

I expose into the area I want to section, then apply a gauze pad soaked in warm water and leave it for a few minutes. The lens will then be rehydrated enough to cooperate. The lens should look like glass in the ribbon.

When it all works well, eyes can be great fun. Tough to believe at this point, probably!

Mary Georger, Astra Arcus USA

A Note On Electron Probe and SEM Filaments And Emission

Electron Probe filaments are just the same as those used in the typical SEM. The difference is the position of the filament. If you place the filament a long way from the cap, you need less heat because the bias field is more effective. The result is less evaporation, less emission current and a longer filament life. You do not need many electrons to generate enough x-rays for analysis compared with normal SEM imaging!

Saturation is saturation; it should be on the plateau of the graph. However the plateau may be moved higher or lower in the heat range depending on the position of the filament and the amount of bias being used. Don't write off electron gun importance. Probe current is basically the number of electrons: get more electrons from the gun and the current goes up. The gun sets the quality of an SEM image. Set the gun up *incorrectly and the rest of the system cannot compensate, you just run out* of electrons!

Driving the filament hard means pushing it forward and increasing the bias field to constrain the beam (aiming in a Japanese instrument for 100 to 120 µA emission current). This filament pushed forward increases the number of electrons being emitted from the cap, but this means more heat is required to reach saturation as more heat is lost to the cathode cap, and because the bias field effect is weakened. More heat shortens the filament life through evaporation. Increasing the bias constrains the electrons, funneling them together to try to achieve a small source. High performance requires a small, highly electron-dense source, which is improved further by using the correct anode-cathode distance of 1 mm for every 2 kV.

Put very simply, a 50 µm source gives a 50 Å microscope, the condenser system giving about a 10,000X reduction. Reduce the source size but keep up the number of electrons it constrains, and you improve the instrument's performance: a 40 µm source gives a 40 Å microscope. It is possible to get more than you paid for, but the cost is filament life.

Steve Chapman, Protrain
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Isopentane Freezing for *In-Situ* Hybridization

For delicate tissues, especially those with a high water content (like soft embryonic tissue tends to be, at least, true for brain) that may be prone to cracking, I recommend freezing in isopentane cooled on dry ice.

Put the isopentane into a glass beaker and bury the beaker up to the liquid level in dry ice chunks.

Let it sit for 10 minutes or so, and I think the temperature is approximately -50° C. A little warmer than liquid N_2 slush, but I think a bit difference in preventing cracking of that delicate tissue.

For our brains (adult rodent as well as embryonic), we have made a little foil "basket" with wire handles, and we dip the tissue in and out of the isopentane a few times before completely immersing it and leaving it for 20 to 30 seconds.

We freeze everything directly upon removal, without sucrose protection, and we generally have no problems with cracking. The cell morphology is, of course, not what I'm used to with perfusion-fixed material.

David Morilak, University of Texas Health Science Center