# NETNOTES

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**Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 9/10/04 to 10/10/04. Postings may have been edited to conserve space or for clarity.**

**FIXATION - Trump's fixative**

*I work for a small research lab and we plan to do TEM on mouse tissue. We will fix the tissue in Trump's which is 4% formaldehyde + 1% glutaraldehyde in a phosphate buffer. Has anyone used this fixative before? Are there any pitfalls that we should avoid? Scott Duong <sduong@bidmc.harvard.edu> 16 Aug 2004*

Phosphate fixes can result in fine calcium precipitates. I prefer HEPES or PIPES. Tom Phillips <phillipst@missouri.edu> 16 Aug 2004

I use a modified Trump's fixative with 0.1 M sodium cacodylate buffer without problems with most kinds of tissue. I also use 0.1 M phosphate buffer without artifact problems, as long as I wash in buffer 3X after fixation prior to osmification (1% osmium in 0.1 M buffer for 60 minutes), followed by 3X rinse in buffer and an additional 3X rinse in water prior to dehydration in ethanol series. My problem had been "osmium peppering" -- a fine precipitate contaminating samples run up in phosphate buffer when not thoroughly washed. Sodium cacodylate avoids this problem, but I always rinse thoroughly when preparing samples for TEM. I run up tissue at room temperature because of a superstition acquired as a graduate student that cold temperature promotes the disassembly of microtubules. Does anyone have thoughts pro or con about running up tissue on ice vs. room temperature? Dean Abel <dean-abel@uiowa.edu> 17 Aug 2004

I have used a combination fixative of 1% glutaraldehyde + 1% OsO4 in 0.05 M phosphate buffer at pH 6.2 on ice and kept in the dark for 45 minutes for many tissues. There are many microtubules present. Perhaps the microtubules were stabilized by the osmium before they were able to disassemble in the cold temperature. The only problem I had with this fixative was with bacteria inside tissue cultured macrophages. It did not wash out as easily/quickly as I expected. As a result, I had the "peppering" of the osmium in and around the bacteria itself when the TEM beam hit the bacteria. Pat Connelly <psconnel@sas.upenn.edu> 17 Aug 2004

**FIXATION – plant leaf**

*I'm working on plant leaf material (sagebrush). The leaf is covered by minute white hairs and due to this reason I'm not able to find the distribution of stomatal cells. Could you please suggest a way to remove these hairs so that I can see stomatal cells? I also want to measure the size and depth of stomatal cells. Could you please tell me the way to fix leaf in its living condition? Kusum Naithani <kn77@uwyo.edu> 31 Aug 2004*

I don't know a good way to remove the hairs, but for counting and measuring stomata, it would be easier if any waxy cuticle was removed during processing. I have done this by using acetone as a dehydrant, rather than ethanol. In my experience, it cleans up the surface of the leaf quite well and makes the stomata and other surface features stand out nicely. Randy Tindall <tindallr@missouri.edu> 1 Sep 2004 You can try removing the trichomes by very gently shaving the leaf. Hold a razor blade almost vertical, just not touching the leaf surface, and pull in the direction of the tilt. This requires a steady hand, and doesn't work on robust trichomes, but might on sagebrush -- I don't know that plant. The other approach would be to go at the leaf with the blade near horizontal and again just not touching the leaf, like shaving your face. You don't need to remove the entire trichome, just most of it, so the surface is revealed. The best way to measure the size of the stomatal cells would be with a light microscope on freshly picked leafs in a room (or chamber) of the appropriate humidity. Or, same conditions but make a replica with dental silicone or one of the replica materials the EM companies sell. The replica could then be examined in the SEM, or with a light microscope. Philip Oshel <peoshel@wisc.edu> 01 Sep 2004

Here is my suggestion for removing trichomes (or root hairs): Follow any standard fixation protocol but you'll find that sagebrush does not "wet" very well, so you'll need to add a surfactant like Kodak Photo-flo or Tween (1 drop / 10 mls fixative is generally enough) - if you are looking to follow up with light microscopy then FAA might work very nicely for you. FAA is a Formalin:Acetic Acid:Alcohol fixative made by mixing 5 mls of formalin with 5 mls of glacial acetic acid and 90 mls of 50% (or 70%) ethanol. The alcohol works well to "wet" the material. In any case, dehydrate the samples to 50%-100% ethanol, plunge freeze in liquid nitrogen, rub the surface of the leaves with a wood stick, pre-cooled razor blade, plastic bar, etc. This should break all the trichomes off the leaf surface revealing the stomata. Transfer samples back from liquid nitrogen and continue with sample prep. To accurately measure stomata depth you will have to section the samples and look at the cells in cross-section. Richard Edelmann <edelmare@muohio.edu> 1 Sep 2004

One possible way to remove hairs is to make a surface replica of the surface using nail varnish or a mounting medium such as Shur Mount and stripping off when dry. On most of the leaf tissues I've worked with it removes hairs, fungi, and surface debris but does not damage the surface itself. Ian Hallett <ihallett@hortresearch.co.nz> 02 Sep 2004

For a simple and effective method for making plant surface replicas for observation by LM or SEM, see my article in the Nov/Dec 2003 issue of Microscopy Today: Cellulose Acetate Replication of Plant Surfaces for SEM. You'll see images of stomata there (http://www.microscopy-today.com). However, if a plant surface is very thickly populated by a tangled mess of hairs, or trichomes, then replication may not be possible, as it will be full of holes left by the trichomes and may tear apart upon attempted removal from the surface. If the hair density is not too high, even though holes from trichomes may be present, you may still be able to see enough surface to get a good sample of the stomata. Gib Ahlstrand <ahlst007@tc.umn.edu> 02 Sep 2004

**FIXATION – Dinoflagellates**

*I'm working with some extremely fastidious dinoflagellates, which I can't seem to keep the flagella in place. I'm after the flagella attachment of the Pyrodinium bahamense. On a separate batch I'm looking to break the armor to take a look inside. I have modified the dehydration process in order to see if that preserves the flagella. I'm also thinking to change the fixative from paraformaldehyde to glutaraldehyde. Any ideas into how to treat these organisms will be greatly appreciated. Omayra Velez <mayas003@yahoo.com> 16 Sep 2004*

Dinoflagellates are not easy to fix properly. My experience with them has been that each species (especially the lightly armored or unarmored ones) can have their own individual requirements for fixation. When I have problems with fixation I use what has been called Parducz fixative. It was originally developed in the 1960's for work on cilliates by Bela Parducz to study the metachronal wave pattern of the cilia. It consists of a mixture of osmium tetroxide and mercuric chloride. To prepare the solution according to Parducz, you make a 6:1(vol:vol) mixture of 2% aqueous osmium tetroxide (OsO4) with saturated aqueous mercuric chloride (HgCl2). You can adjust the relative mixtures of the two components as I know that I have used 3:2 and perhaps 1:1. This fixative has been referred to as an "instantaneous fix" as it will fix the specimens very rapidly. Parducz fixative was the only way that I could prepare some dinoflagellates for SEM and retain not only their morphology, but the flagella as well. It is not as good a fix for TEM. Here are a couple of references that you may want to see: Small (1968) Scanning electron microscopy of fixed, frozen and dried protozoa. Science 163:1064-1065 and Parducz (1966) Ciliary movement and coordination in cilliates. International Review of Cytology 21:91-128. Greg Strout <gstrout@ou.edu> 16 Sep 2004

# Microscopy AND Microanalysis

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We have used Parducz's fix since 1967 and it works wonders on bacteria, protozoa, and many other critters especially those with flagella and cilia. Two things that we have found important in its use are: 1. Make it fresh just before use. We keep a saturated solution of HgCl2 around in a brown 50 ml bottle which has been as old as one year. To make it, we put a bit of HgCl2 into distilled water until we see that it is saturated (i.e., with a precipitate on the bottom). We also keep a bottle of 2% aqueous OsO4 in the refrigerator (usually double jarred to prevent vapors from escaping). Just before use, we mix 6 ml of 2% OsO4 and 1 ml of the saturated HgCl2 (taken from the top of the bottle). We then fix for an hour or so. 2. Wash well after the Parducz fixation. Otherwise you get starfish shaped crystals on the surface of your preparation. They may look neat but obviously are not part of the specimen. Other than that, it is a great hardening fix for SEM. Judy Murphy <murphyjudy@comcast.net> 16 Sep 2004

**EMBEDDING: DMP-30 vs. BDMA**

*Can one substitute BDMA for DMP-30 in a Luft-based epoxy resin? If so, what would the proportion be? Currently, I add 2 ml DMP-30 to 100 ml resin (PolyBed 812, NMA, DDSA). Walt Bobrowski <walter.bobrowski@pfizer.com> 2 Sep 2004*

After years of hearing and reading that BDMA is better than DMP-30, I switched to BDMA. I used it at about 60-80% of the weight of DMP-30 and got equivalent results using freshly made resin mixes. But I saw a difference when I stored my extra resin at -20 °C. The BDMA mixtures became much more viscous (presumably partially polymerized) after 1-2 weeks at -20 °C compared to mixtures with DMP-30. I have gone back to using DMP-30. Whichever one you use, I strongly advise you begin to dispense it and the other components by weight. I have a scale in my fume hood and make 40 gm batches of epoxy resins this way and they are much more consistent and the mess is significantly less. I freeze the complete resin fully mixed with DMP-30. Generally I make aliquots so they are only thawed once. They are certainly good for a couple of weeks this way and longer in many cases. I make all my resin by weight (typically 20 g Embed 812 or its equivalent, 10 g DDSA, 10 g NMA, and 0.6 g DMP-30) in a 50 ml plastic disposable tube and shake vigorously until well mixed. We used 0.8 g BDMA in place of DMP-30 in this formulation and saw no difference in cutting quality but did have the storage problem. The viscosity of the DDSA and NMA and Embed 812 is high and requires vigorous shaking regardless of the catalyst so I don't see the lower viscosity of BDMA as significant if you are measuring by weight. If you measure by volume, a viscous solution will be tougher to accurately measure and deliver and the percent error will be much higher for the small volume BDMA or DMP-30 component. BDMA is frequently touted as superior due to its lower viscosity and less hydrophilic. It has a lower viscosity but I don't see that as a problem or benefit. I have no data on the relative hydroscopic properties but I have never had a bottle of DMP-30 go bad on me in the 25 years I have been doing TEM. Tom Phillips <phillipst@missouri.edu> 02 Sep 2004 and 03 Sep 2004

The only reason that DMP-30 is still around is tradition; by all means substitute the same amount of BDMA. Viscosities: BDMA, 0.85 cP, DMP-30, 20.5 cP. The quantity that you use is small, so there won't be a big change in the viscosity of the mix, but DMP-30 is so viscous that it can actually partition out of the mix during infiltration! By partitioning out, I mean that it doesn't enter the tissue as rapidly as the other resin components. The symptom is soft tissue in a normal, hard block. DMP-30 is hygroscopic, which leads to more problems. The original reference is A. Glauert, Proc. RMS 22:264 (1987) and you'll find the data in chapter 6 of Glauert & Lewis, Biological Specimen Preparation for Transmission Electron Microscopy, Princeton, 1998. When I was still teaching in Berkeley, I could always cure Epon problems by having the investigator switch to BDMA. Caroline Schooley <schooley@mcn.org> 02 Sep 2004 and 03 Sep 2004

For a less viscous mixture, you could try one percent DMP-30 instead of two percent. The final cure might not be quite so hard, though, so depending on your tissue this may or may not help. Lesley Weston <lesley@vancouverbc.net> 03 Sep 2004

**EMBEDING – Infiltration problems**

*I processed quite a few blocks of tissue into Spurr's resin last week. Some of the tissue was actually cell pellets embedded in conical capsules. These blocks and some others in regular capsules did not polymerize in the 2 days as I expected. I left them in the oven over the Holiday, but they are still soft. I may have made the Spurr's wrong. Does anyone know if these will eventually cure or can I dig out the tissue, put it into a Spurr's/propylene oxide mixture and try to re-embed it? Of course these were the most important blocks in all that I embedded! Thanks. Stacey Andringa <stacey.andringa@uc.edu> 7 Sep 2004*

It's unlikely that the samples will polymerize much more after a couple of days. You don't say whether you use Spurr's regularly and this is a first batch to go wrong. You can certainly try re-embedding but be prepared for a slightly poorer block. Spurr's can poorly embed for a variety of reasons: 1. Incomplete dehydration or incomplete removal of alcohol - although if you're using propylene oxide as well I wouldn't have thought so. 2. Incomplete impregnation with resin because too little time or not enough stages (e.g. 50%, 75%, 2x100%) or not enough agitation/rotation. 3. A bad mix either due to wrong amounts or incomplete mix. 4. One of the components has deteriorated (The S1 curing agent may have a shelf life of 6-12 months; I believe that the NSA anhydride hardener can go off especially if exposed to moisture over time). Spurr's can be affected by moisture so if you chill or freeze it, allow plenty of time for it to reach room temperature and don't leave the lids off the components or mixture for too long. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 08 Sep 2004

**EMBEDDING – Infiltration problems due to humidity**

*It seems that during the summer months, we have no end of troubles with our plastic not infiltrating the tissue. I've always assumed that this was because of the humidity rising quite a bit during the summer months, and the poor air conditioning. It comes out as sections falling apart when I try to flatten them in the water boat, or breaking in the electron beam. We have always kept our epoxy resin in the freezer in 30 ml plastic syringes, but it usually only gives us a problem during the summer months. During the winter here, the air is extremely dry. We do 4 rinses in absolute alcohol, followed by 3 rinses in propylene oxide, followed by a 50/50 mixture of Epon/Araldite mixed with propylene oxide over night with the caps off, with the propylene oxide evaporating overnight and slowly leaving the specimens in 100% plastic by morning. We polymerize at 70 °C overnight, keeping a desiccant in our embedding oven to keep the air as dry as possible. Are there any resins that might be used for electron microscopy that are more tolerant of water, and would give a good result with human tissue in the electron microscope? Garry Burgess <gburgess@exchange.hsc.mb.ca> 13 Sep 2004*

Your procedure is similar to the one I use here in Houston where we also have very high humidity. The difference is that my Epon/Araldite: propylene oxide step is for one hour followed by one hour in pure resin then embedded in capsules in fresh resin. This sits at room temperature for 3-4 hours then in the oven overnight at 80 °C. I have been using this method for over 20 years with no problems. Mannie Steglich <msteglic@mdanderson.org> 13 Sep 2004

If the problem is caused by high humidity in the air, you could try using a water-miscible resin such as Durcupan or Aquembed. They're both a lot more expensive than Epon-substitutes and Araldite and they polymerize to a rather soft block, which may or may not matter depending on your tissue, but it would eliminate the problem if that is the cause. Another possibility is that there might still be a trace of propylene oxide left, if you don't do one more 100% in the morning before transferring to the DMP-30 mix, but I don't know why that would happen only in summer. Lesley Weston <lesley@vancouverbc.net> 15 Sep 2004

I am chronically plagued by humidity problems here. Besides making sure the bottles of absolute ethanol, propylene oxide and resin components are opened as little as possible, I put my vials with samples

over desiccant whenever I'm using absolute ethanol, propylene oxide, or resin. I have a rotator we made years ago that accommodates film cans with desiccant into which my vials fit. I keep desiccant in the embedding oven. And, importantly, I pre-heat my molds and labels overnight in the oven before putting in the samples. This preheating of the capsules or molds and paper labels also keep bubbles from forming so that I never have to pull a vacuum on them. Tina Carvalho <tina@pbrc.hawaii.edu> 15 Sep 2004

*How effective is molecular sieve as a desiccant at typical polymerization temperatures (60-70 ºC)? I would also be interested to know what strategies people now recommend for drying EM solvents without getting them contaminated with bits of desiccant. How can one determine whether the desiccant is still being effective? Can you trust the blue indicator in molecular sieve? What is the equilibrium water concentration in ethanol above fresh molecular sieve (assuming the ethanol contained some water initially), and does this increase when the molecular sieve contains, say, 10% water (half its saturation capacity)? Chris Jeffree <c.jeffree@ed.ac.uk> 15 Sep 2004*

To avoid the bits in your solvents, try putting your molecular sieve into a length of dialysis tubing. You can fold the ends over and staple them to form a small sausage. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 15 Sep 2004

Presumably the dialysis tubing has to be wet before it can be loaded. Can you re-dry the molecular sieve after loading? Chris Jeffree <c.jeffree@ed.ac.uk> 15 Sep 2004

Yes it has to be wetted, but just wet the end and pour the sieve in with a funnel. Trim off the wet part and either tie or staple the ends. As for working, I also make a little packet of cupric sulfate the same way as an indicator and it turns blue indicating it has taken up moisture. Dry cupric sulfate over a flame until it turns white. Recharge when blue. I generally need to do this once a year up here. All my 100% solutions (ethanol, acetone, and HMDS) are on sieve. Scott Whittaker <whittaker.scott@nmnh.si.edu> 15 Sep 2004

We have been using molecular sieve in dialysis tubing for at least 20 years as a means of drying our 100% ethanol and acetone. Note that I am in Florida where the humidity is quite high for many months out of the year. Dry dialysis tubing can be opened, in order to fill it, by getting a small opening at one end and then blowing air through it to force it open all the way. A little blue indicator silica gel can be added for ethanol, but not acetone, since the dye is acetone soluble. Greg Erdos <gwe@biotech.ufl.edu> 15 Sep 2004

I use acetone for my dehydrations (prior to Spurr's infiltration). My professor taught me years ago to dehydrate acetone with CuSO4. Place about 50-100 g of CuSO4 in an evaporating dish in a muffle furnace for about 6 hrs. The deep aqua crystals will turn to a white powder with a faint greenish cast. Allow to cool slightly (in the furnace), add to an empty bottle, add "100%" acetone from a freshly opened 500 mls bottle, shake, and allow to settle over night. As long as the copper sulfate does not change color, the acetone is assumed to be adequately desiccated. I've used this with complete success for acetone, with not apparent affect on specimens from the copper. I have no idea whether it works for ethanol. Donald Lovett <lovett@tcnj.edu> 15 Sep 2004

We have quit using molecular sieves just in case they were responsible for what we thought was excessive wear on our diamond knives. Since we do a lot of microwave processing, we generally use acetone, rather than ethanol, and mix our dehydration series fresh each time. When I use ethanol, I usually finish up with a recently opened bottle of absolute, but after two or three uses I relegate that bottle to the 90-95% category, and open up a new one for the final steps. This seems to work for us, as we rarely have infiltration problems, even with retinal tissue which can be problematic. Randy Tindall <tindallr@missouri.edu> 15 Sep 2004

On the subject of humidity causing poor infiltration: several of my colleagues go from 95% ethanol to Epon substitutes without any problems. No absolute alcohol, no propylene oxide. Yes, extra changes of epoxy are needed but the results are fine. Hayat's Principles and Techniques of Electron Microscopy, 2nd ed., vol. 1, page 154 reports that Epon is miscible with 70% ethanol. I was "raised" with the "you must get every last molecule of water out of the specimen" dogma but experience had taught me otherwise. I suggest you look elsewhere for the cause of your difficulties. Geoff McAuliffe <mcauliff@umdnj.edu> 15 Sep 2004

In dense plant tissues, we generally get problems in embedded tissues not because of incomplete dehydration, but due to infiltration either too fast or in steps that are too large. If cell walls are moderately impermeable, then solvent may diffuse out of the tissue faster than resin can diffuse in, causing tissue collapse and "airspaces" when remaining traces of solvent within such tissue vaporize during polymerization. Slower infiltration, with smaller increases of percent resin in solvent, and holding the tissue longer at each step, has generally solved this problem, for us, anyway. As an example, a friend found that the single- to few-celled algal zygotes she was working on had to be infiltrated in increments of 1-2% resin per day up to 10% or so, after which the increments could be larger, then above 90% resin, the increments had to be smaller again. If this wasn't done, the zygotes looked like flat balloons after resin polymerization. Not sure if this applies to animal tissues but is another protocol modification to try. Rosemary White <rosemary.white@csiro.au> 16 Sep 2004

**EMBEDDING – LR White**

*The following response was posted in reply to a question about flat embedding capsules that excluded oxygen for use with LR White embedding:*

I used to cut the tips off the polyethylene embedding capsules and place an extra cap on the cut end. This would give me a cylinder with

two end caps. The block would be a little shorter than the standard capsule. Joel Sheffield <jbs@temple.edu> 22 Aug 2004

**LM - stigmatism problem or auto focusing problem at high magnification**

*Does anyone know whether there is stigmatism problem or auto focusing problem at high resolution on an optical microscope? <kssim@mmu.edu.my> 19 Sep 2004*

A nice introduction to astigmatism in microscopy can be found at: http://micro.magnet.fsu.edu/primer/java/aberrations/astigmatism/. Autofocusing on a digital microscope works fine, even at high magnification (63x, 1.4 NA) in both brightfield and fluorescence microscopy, as was published in: Geusebroek J.M., Cornelissen F., Smeulders A. W. M., and Geerts H. (2000) Robust autofocusing in microscopy. Cytometry 39(1):1-9. Regardless of which algorithm you use, it is important to sample your Z-stack at the appropriate interval (see Geusebroek et al.). Peter Van Osta <pvosta@maia-scientific.com> 20 Sep 2004

Peter Van Osta provided a reference to a very helpful article. I also found the following article helpful: Santos et al. (1997) Evaluation of autofocus functions in molecular cytogenic analysis. J. Microsc., 188(3):264-272. In particular, I found the functions "F4" and "F5", attributed to Vollath, to be robust. John Minter <jrminter@rochester.rr.com> 20 Sep 2004

**LM – section wrinkling**

*Recently, I have been having problems with my thick sections lying flat. Nothing has been changed in the protocol; section, stain, and rinse. No matter how carefully I place the section on the slide, it will not be flat after the slide has dried. Lesley Graham <patljg@gwumc.edu> 21 Sep 2004*

I presume that you have already stained your semi-thin sections at that point, when you notice that the section is no longer flat. One thing that I have noticed is that a person needs to wait a bit longer after the section has dried on the hot plate before they start staining. So when the section is drying, I am busy typing my slide labels, but even then, when the slide looks dry and ready to go, it isn't in fact ready to go, and you need to give it a few more minutes, otherwise, you won't have a good bond of the section to the slide, and it will start to lift off during the staining, and especially the washing after the staining. This could be the most frustrating thing in the universe if this happens. If on the other hand, you give your slide some time to bond to the slide, (a few minutes) even after it has dried down, then the sections will refrain from lifting up and folding over during the staining process. If you allow it this extra time, you can be quite rough with the slide during the staining and washing and those sections won't lift off and fold. And not only that, but you won't have to use coated slides to make sure that the sections won't wash off, because they won't be going anywhere. Garry Burgess <gburgess@exchange.hsc.mb.ca> 21 Sep 2004

When I cut resin thick sections (0.5 - 5.0 microns) I transfer the sections to a drop of water on a slide and set the slide on a slide warmer under a large Petri dish cover with a cotton swab dipped in toluene. The toluene vapor softens and smoothes the resin sections so that they flatten and adhere to the slide. Dean Abel <dean-abel@uiowa.edu> 21 Sep 2004

**TEM: Grids continue to break under the 60kV beam**

*My Pioloform coated slot grids continue to break under the 60 kV TEM beam. I cut and look at serial thin sections and we note that the Pioloform usually starts to break between two adjacent sections. We use a lot of liquid Nitrogen in the scope and it helps somewhat, but we still get breakage. I understand at 60 kV the electrons are slower and are heating up the grid, compared to working at higher kV's but because of our low contrast brain tissue, we are forced to work at 60 kV. I could try re-coating the grids with a thinner layer of Pioloform but that has some risks and lowers resolution somewhat. We also don't have a carbon coater and choose not to carbon coat because of the risks of messing up our series. Knowing all this, could someone suggest another way we can solve our problem? Marcia Feinberg <mfein@bu.edu> 31 Aug 2004*

You need to conduct heat and charge away from the spot where the electron beam interacts with the specimen, and, since you do not want to coat the specimen and the Pioloform is not a good conductor, a remaining possibility is to lower the dose rate to the point that the heat and charge will dissipate. Possibly, pre-irradiation of the entire slot with a low dose could produce enough conductivity in the Pioloform to overcome the problem. I'd experiment with grids that do not have valuable material on them to determine what pre-irradiation and/or dose rate are optimal. Bill Tivol <tivol@caltech.edu> 31 Aug 2004

Some thoughts on your breaking grids: 1. Make sure the Pioloform is absolutely fresh. 2. Can you use a regular mesh grid for additional support? 3. You could obtain carbon coated slot grids from an EM supplier. John Arnott <ladres@worldnet.att.net> 1 Sep 2004

**TEM – bright spot**

*When we use our TEM at low magnification (below 4,000X), we sometimes get a bright spot in the middle of pictures we take. I always thought the microscope setting was the only thing responsible for this problem. But when I checked into a latest episode, I found pictures from one particular specimen (thin sections on Formvar coated grid) had it, but others from a different specimen (negative staining on Formvar/carbon coated grid) did not, even though all pictures were taken with the same scope setting. Has anyone else experienced the same? Can anyone explain to me thoroughly how this problem occurs, and how to avoid it? Hong Yi <hyi@emory.edu> 21 Sep 2004*

The most likely cause is removal of the plastic due to the beam -- resulting in an area of lowered density. This is quite common (and can sometimes be used to enhance the contrast of sectioned specimens). What is probably happening is that someone is investigating the specimen at high magnification with the beam condensed to a small spot. When you then go to a lower magnification, you will see the area where the beam spot has etched away the plastic. Also, some people focus the image with the condenser reduced in size (since it is much brighter). If too much time is spent in this condition, you will see the etching of the plastic after the beam is spread. One work-around would be to record the images at low magnification first and then do the high magnification work (where the condenser is reduced in size) and avoid focusing at crossover. We sometimes use this with specimens of low contrast (LR White and Spurr's, for example). As the plastic is removed by the beam, the remaining specimen appears with greater contrast. In fact, our TEM even has this capability programmed in by the manufacturer (the beam is traversed over the specimen to "stabilize" the plastic--and it also improves contrast). John J. Bozzola <bozzola@siu.edu> 22 Sep 2004

Thank you for your input. I definitely have seen this removal of the plastic phenomenon on scope. But I am not convinced that is all that was happening here. The spot was there even if we moved away from etched area. Also, the spot sometimes was so intense that it could be seen on phosphor screen. Someone suggested that it has something to do with the distance between the pole piece and the objective aperture. I think I agree, but it seemed that the type of sample prep played a role in it too. Hong Yi <hyi@emory.edu> 23 Sep 2004

I knew what you were referring to on the low power magnification and just checked it out on my old Philips 200. The spot is evident on the screen when in low magnification and hence would be on the negative if I took a picture. It even shows up on the grid bar when I scan only when the image is out of focus! If you have a wobbler on your scope use it to get into focus and see if the light spot disappears. If you do not have a wobbler, put in a holey grid or of course a thicker section that has a hole or two and focus on the hole to the point where it is in focus and look for the spot. I bet it is no longer there. Pat Connelly <psconnel@sas.upenn.edu> 23 Sep 2004

**SEM – critical point drying with isoamyl acetate**

*Some protocols for SEM indicate that following ethanol series dehydration, samples should be soaked in isoamyl acetate and that this media*

*should also be used for critical point drying. Can anyone tell me what the theory is behind using isoamyl acetate and what benefit, if any, there is over leaving samples in 100% ethanol prior to the critical point drying? Mark Evans <mark.evans@uvm.edu> 29 Sep 2004*

I have not used amyl acetate for critical point drying (CPD) since my first CPD run almost 30 years ago. The head of department was incensed by the strong pear/banana smell and banned it as antisocial. In those days nobody seemed to worry about its toxicity. Amyl acetate also damages the rubber seals of the CPD faster than other solvents. The objective is to fully replace the dehydrating solvent with liquid CO2. Amyl acetate is more soluble in liquid CO2 than the more polar ethanol, but many people nevertheless use ethanol routinely and obtain great results, especially if the specimens are small, thin, and unenclosed by cuticle (cultured cells on coverslips, for example). If the specimens are large chunks of plant material it would be better to work with acetone (propanone) as both dehydrating and intermediate solvent. Chris Jeffree <c.jeffree@ed.ac.uk> 30 Sep 2004

Amyl acetate is supposed to be more miscible in liquid CO2 than is ethanol. I have also heard that it's supposed to be a suspected carcinogen, but I don't have any references on that. One reason I was given for using amyl acetate when I was learning EM was that it smells of bananas, and therefore it can be easily known when it's all been flushed out. I've never used amyl acetate, just ethanol, and I get fine results. I may have to purge more than I would if I used amyl acetate, but not much. And the ethanol has plenty of odor, so it's also easy to tell when the bulk is gone by smell. But it's the amyl acetate or ethanol in the samples that has to be gotten rid of, not the bulk fluid, and that's too small a volume to smell anyway. Philip Oshel <peoshel@wisc.edu> 30 Sep 2004

The very old Sorvall Critical Point Dryer came with a spare parts box that had special O-rings that needed to be used if amyl acetate was to be used. The directions did not give any reason for using it. I have usually used acetone for dehydrations and a freshly opened bottle of acetone for the final exchange before the drying. Many years ago an investigator insisted on using amyl acetate with his sample and he ran the operation himself. He later admitted that similar samples that I ran in acetone looked better. If one uses amyl acetate in the critical point dryer it is extremely necessary for the amyl acetate to be properly vented into a hood (acetone also). If the amyl acetate of this volume gets into the room it can make one behave strangely. My co-workers thought that I was drunk! I did not make that mistake again. Pat Connelly <psconnel@sas.upenn.edu> 30 Sep 2004

**SEM – image darkening effect**

*Can anybody comment on the image darkening effect as one progressively scans the surface of a sample with a SEM? What are the possible physical causes, and how can you reduce the effect? Pei Zou <pzou@feico.com> 01 Sep 2004*

See section 9.10.6 in Goldstein et al. "Scanning electron microscopy and X-ray analysis" Plenum Press 1992. It begins: "A sample subjected to electron bombardment in a diffusion-pumped vacuum gradually becomes covered with a contamination layer due to polymerization, under the action of the beam, of organic matter adsorbed on the surface". Ways to reduce the effect are: clean vacuum, clean sample, cold finger. Jorgen Bilde <j.bilde@risoe.dk> 02 Sep 2004

**EDS – non-carbon nanotubes**

*Recently, we tried EDS analysis of non-carbon nanotubes (20-30 nm in diameter) in a TEM microscope (Tecnai F20). The signals from compositional elements are very tiny in comparison with the copper and carbon signals, which makes perfect sense, because we used carbon coated grids. However, in the literature such EDS spectra from a spot in one (not from a bunch), say a ZnO nanotube exhibits signals comparable to those from Cu-C background. How it is possible, that people obtain so high intensity of compositional elements from a very small analyzed area? What should we do to improve our results of getting EDS from a single nanotube? Is it necessary to use beryllium grids? Andrei Burnin <burnin1970@hotmail.com> 11 Sep 2004*

The subject of EDS spectra in a TEM or STEM is complex and there are many different configurations of EDS detector in the TEM column. Some of these account for the differences in relative background and specific element signal. If you have a horizontal-mount detector and then tilt the sample towards the detector, this will pick up a lot of grid and carbon x-rays. I have a TEM with a high-takeoff-angle detector (68 degrees) and that results in a much lower Cu and C signal when looking at tiny particles on a carbon-coated copper grid. A 30 $mm^2$ detector will also pick up more counts than a 10 $mm^2$. The material in the specimen holder and the column above and below the sample also affects the relative background of your spectrum. A TEM really needs to be designed for EDS at the factory, with light-element inserts to reduce the secondary x-ray radiation that generates much of your background. The better spectra you are referring to may have come from a TEM or STEM designed or modified to give the best EDS performance, perhaps at the cost of less performance in other areas. I know my TEM was designed for analytical work, at the sacrifice of ultimate resolution. To increase your counts you can try lowering the accelerating voltage, increasing the spot size, increasing the condenser aperture size and, of course, increasing the time you count for. Make sure your objective aperture is removed, the specimen holder is specified for EDS and your EDS detector is properly aligned. Mary Mager <mager@interchange.ubc.ca> 13 Sep 2004

**Material Science – polyurethane polymers**

*I'm looking for a staining method to prepare an ultramicrotomed polyurethane polymer for a TEM investigation. Contrast is needed between the hard and soft segments of the polymer. The functional groups of the soft segments are mainly carbonates, and of the hard segments amides or urea. Both segments also have a different urethane content. Are there staining methods recommended for polyurethane polymers with such a combination of functional groups (for certain urethanes, OsO4 or RuO4 may be used)? Can such polymers be imaged by coherent phase contrast alone? Thomas Keller <t.keller@uni-jena.de> 17 Aug 2004*

Depending on the size of "soft" and "hard" regions, the best way to image them can be variants of phase-sensitive AFM (phase mode), force modulation mode, or acoustic or ultrasonic force microscopy. Basically, all these techniques are sensitive to hardness and losses at the tip-surface junction; the variation is primarily in frequency range and modulation/detection mechanism. Resolution is primarily limited by the tip-surface contact area and can be as small as few nanometers. Sergei V. Kalinin <sergei2@ornl.gov> 17 Aug 2004





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