NETNOTES

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Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 04/15/06 to 06/15/07. Postings may have been edited to conserve space or for clarity.

SPECIMEN PREPARATION - cell cultures and aldehyde fixatives

I'm processing some monolayer cell cultures grown on Thermanox coverslips for SEM. I seem to recall that aldehyde fixatives can cause an artifact that result in small holes in the cell membrane. If I remember, picric acid is added to the fixative and that it helps prevent the artifact. I don't remember where I originally read this. Am I off base on this? If it is correct, does anyone remember the formulation of the fixative using picric acid? I currently use 2% glutaraldehyde, 2% paraformaldehyde and 0.5% acrolein in 0.1 M Sorensen's phosphate buffer, pH 7.2. Also during processing the thin cytoplasmic extensions of the cells break. I assume this is due to shrinkage during the dehydration steps and critical point drying. Anyone know of a way to prevent this? Tom Bargar <tbargar@unmc.edu> 16 Apr 2007

For the membrane holes, try 1% tannic acid in the glut. You want the monomeric form, Mallinckrodt 1674 - or 1764, I keep transposing those digits. 1% tannic acid can also be used in an OsO_4 post-fix. I would also suggest cutting the glutaraldehyde to 1 or 1.25%, and don't bother with the formalin, it's not need for cell monolayers. Acrolein I haven't used, so can't comment. I've just done 1-1.25% glutaraldehyde + 1% tannic acid. I don't know about picric acid, and would be interested to hear if it works. Phil Oshel <oshel1pe@cmich.edu> 16 Apr 2007

I use picric acid in my primary fix to preserve membranes. My "recipe" is 2.5% glut, 4% paraformaldehyde in 0.1M Na-cacodylate with 0.02% picric acid. I did the math years ago, and this worked out to be: 1 x 10 ml vial of 10% glut + 1 x 10 ml vial of 16% paraformaldehyde in 20 ml of 0.2m cacodylate with 2 ml of saturated aqueous picric acid added. I've had the same 100g bottle of picric acid for over 15 years. I just keep it saturated with the liquid well above the level of the crystals. When I draw some off, I add more water. Our Life Safety guys here are just thrilled with me: osmium, uranium picric acid, suspected carcinogens. As long as you are careful about keeping the crystals fully under water, and not letting any accumulate around the rim or anywhere where they could dry out, you're fine. As for your broken processes: they may be getting beaten up during your CPD run. Be sure to do your CO_2 exchanges gently, never letting the fluid level drop below your sample (this will mean extra exchanges), and then, at the end, vent very slowly...100 psi/minute or less. Leona Cohen-Gould <lcgould@med.cornell.edu> 16 Apr 2007

SPECIMEN PREPARATION - stabilization of membranes

A few days ago I posted a query for suggestions on how to stabilize lysosomal membranes in cultured cells prior to immunocytochemistry. We have tried several of the tips but without success. Thanks to those who replied! We have been following the condition of the lysosomes using a GFP tag that we know to be specifically compartmentalized. We note that following fixation and a rinse in TRIS-Glycine the GFP is still compartmentalized. However, perhaps in 90% ethanol and definitely in 1:1 LR White:90% ethanol we see the GFP moves from the lysosomes to the cytoplasm and nucleus. It seems that ethanol and certainly LR White contribute to the deterioration of membranes. Can anyone suggest an embedding media that might be a little gentler on membranes and still possibly work for surface label immunocytochemistry? We are thinking of trying Nanoplast, a water soluble media but any suggestions are welcome. Thanks! Doug Douglas R. Keene <drk@shcc.org> 18 Apr 2007

Have you attempted immunocytochemistry following routine aldehyde fixation and osmium post-fixation followed by standard epoxy embedding? The osmium will preserve the membranes and you may still get successful immunocytochemistry. Give a holler and I'll send you a (work in progress) protocol that appears to yield successful immunohistochemistry on LM thick sections using room temperature reagents (no microwaves, no heated steam baths). Walter Bobrowski <walter.bobrowski@pfizer.com> 20 Apr 2007

Many thanks to those who responded to my request for suggestions for stabilizing membranes (lysosomes in particular). My specific aim in this experiment is to stabilize cultured cells containing GFP tagged lysosomes for surface-label immunocytochemistry with the hope of co-localizing GFP expressing protein (using confocal images overlaid onto TEM images cut from the next serial section) and another potentially interactive protein localized with immunogold on the TEM section. We are able to follow GFP emission through the protocol using our confocal microscope. In cultures fixed in media buffered 4% paraformaldehyde/1% glutaraldehyde, we see that GFP remains compartmentalized up to 90% ethanol but after the introduction of 1:1 90% ethanol:LRWhite, GFP is no longer compartmentalized and instead is distributed throughout the cytoplasm and nucleus. I am now in the process of trying some of the ideas. A preliminary result suggests that the lysosomes are stabilized somewhat more by the inclusion of 4% picric acid; also by progressively lowering temperature during graded ethanol dehydration to -20ºC, embedding in Lowicryl HM20 at -20ºC, and polymerization at -20ºC. We have yet to section the HM20, but we hope that confocal microscopy on 0.5um sectioned HM20 will reveal compartmentalized GFP. Then we'll see what mess we've made of the additional antibody-binding epitopes. We love a challenge! To address the request that the responses be shared, here is a summary. Since some of these responses were made privately, I have not included the author's names. Response #1: what may help: high-pressure immobilization (cryo-fixation; expensive but really good), followed by freeze-substitution at -90ºC(8 to 48 hrs)/-60ºC (8hrs)/-30ºC (6 to 8 hrs) as described in several papers. It is worth trying: Acetone + 0.1/0.2% OsO4, + 0.5% uranyl acetate ± glutaraldehyde (0.1 to 1%) ± formaldehyde (0.25 to 2%) (many variants possible, I know) or ethanol, no OsO4, but add some or all other chemicals/fixatives methanol, no OsO4, but add some or all ... Acetone + 2%GA (ready available as it is) Acetone + 0.2% glutaraldehyde + 0.5 % uranyl acetate ... and so on. The advantage of cryo-substitution at very low temperatures: The chemicals are not reactive at low temperature initially (only slowly at -60ºC, and then at higher temperatures). The chemicals become evenly distributed

in the cell/tissue, and then react at the same time upon warming, everywhere, similarly. Result: tissue, cells and membranes are often better preserved. in addition, you may add some water (1 - 5%?) to the freeze-substitution medium, as suggested by Paul Walther a few years ago. We have done this, others as well, with success. Response #2: Have you tried tannic acid! It is supposed to stabilize plasma membranes, don't know about Lysosomal membranes, Response #3 : While it would involve doing freeze-substitution, Lowicryl HM 20 is great for membranes. You would need to do low temperature dehydration after room temperature or 4ºC aldehyde fixation. I used to use a small amount of glutaraldehyde with the methanol free buffered formalin. I also used uranyl acetate to help preserve membranes and dehydrated cold with methanol. You can look at my paper for details: The Journal of Histochemistry and Cytochemistry 40(10):1491-1500, 1992 "Immunocytochemical Localization of Lysozyme and Surfactant Protein A in Rat Type II Cells and Extracellular Surfactant". Response #4: I have a couple of thoughts on the problem. I don't think you are getting any buffering capacity from the culture medium. And if there is any serum in the medium, you have titrated all the aldehydes with the serum and have no functional fixative left when you are trying to fix the cells. Summary of additional responses: These included longer fixation times, fixation at ambient temperature not 4ºC, and the importance of including Calcium in the fixation buffer. Again, thank you to those who responded and to all interested microscopists! Doug Keene <drk@SHCC.org> 20 Apr 2007

SPECIMEN PREPARATION - lung tissue

Without using a microwave, freezing or perfusion of the tissue; is there a "really" good procedure for fixing pieces of animal lung tissue that someone would be gracious enough to share? Ed Knoppel <eknoppel@cc.usu.edu> 19 Apr 2007

There is a method that works really well in fetal mouse lung. (Cole TJ, Solomon NM, van Driel R, Monk JA, Bird D, Richardson SJ, Dilley RJ, Hooper SB. Am J Respir Cell Mol Biol 2004 May; 30(5):613-9 Altered epithelial proportions in the fetal lung of glucocorticoid receptor null mice.) We used the method as published by Williams, M. C. 1977. "Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs". J. Cell Biol. 72:260-277. It involves use of veronal (barbitone) and maleate buffers, but is simple to do. However, I do not know how well it works with inflated lungs. Briefly: Dissect lungs from embryo, place in drop of fixative and slice gently into mm cubes. Fix in 4% paraformaldehyde + 2% glutaraldehyde + 4% sucrose in HEPES buffered saline pH 7.4 for 3 to 4 hours at room temp. Rinse 2 min in cold veronal acetate, pH 7.4. Postfix in 1.5% OsO_4 in Veronal acetate at 4ºC overnight Rinse 3 x 10 min at 4ºC in Tris Maleate pH 5.2 En bloc stain with 1.5% uranyl acetate in Tris Maleate pH 5.2, 90 min on ice, in dark, cold dehydration, 5 minute changes in graded acetones on ice (10, 20, 30, 40, 50, 60, 70, 80, 90, 95%) Then absolute dry acetone 4 x 10 minutes, the last 2 changes at room temperature. Absolute acetone:Epon mix, 50:50, 30 minutes, rotating Epon, 3 x 60 minute changes, can leave one change overnight, rotating embed in fresh Epon, polymerize overnight at 60 to 65ºC. If you would like the buffer recipes, let me know. Rosemary van Driel <rosey.vandriel@csiro.au> 20 Apr 2007

SPECIMEN PREPARATION – enhancing contrast of mitochondria

Anyone out there have any advice on how to enhance the contrast and definition of the membranes of the cristae of mitochondria? The samples brought to me are monolayer cell cultures of cancer cells grown on Thermanox coverslips. This is how I'm currently processing the samples: Primary fixation is 2% glutaraldehyde, 2% paraformaldehyde, 0.5% acrolein in 0.1M Sorensen's phosphate buffer pH 7.2. Post-fixation in 1% osmium tetroxide in 0.1M Sorensen's phosphate buffer. Dehydration in 50, 70, 90, 95, 100% x 3 ethanol solutions. Embedding in Araldite. Sections are stained with 2% uranyl acetate aqueous 15 minutes and Reynolds' lead citrate 10 minutes. The density of the cytoplasm and mitochondrial matrix are similar with the result that the contrast of the mitochondria is similar to the cytoplasm. The mitochondria and it's membranes (outer and that of the cristae) don't really "stand out". The researchers involved want to see contrasty mitochondrial cristae. The next thing I'm going to try is post-fixation with a mix of osmium tetroxide and potassium ferrocyanide. Tom Bargar <tbargar@unmc.edu> 01 May 2007

You anticipated one of my responses by stating that you are going to use K-Ferrocyanide with the osmium. Another thing you can try (along with the Os/K-ferrocyanide) is to use the following as your primary fix: 4% paraformaldehyde, 2.5% glutaraldehyde, 0.002% picric acid in sodium cacodylate buffer (original ref: Ito & Karnovsky J Cell Biol Vol 89 Abstract #418, 1968). I was first told about this fix by a group who studied outer rod segments of the eye....lots of membranes! Leona Cohen-Gould <lcgould@med.cornell.edu> 01 May 2007

Try an ethanolic-based UA stain: Mix in the following proportions: 0.2g UA 3ml 50% ethanol Let mix at least several hours on rocker/shaker. Next day, try a series of UA staining times and examine (say, every 5 minutes). I've found 10-15 minutes optimal, as longer times appeared to also darken surrounding plastic matrix. Pick your optimal time, stain, rinse, and counterstain with Reynold's LC, ~5-10 minutes, rinse. Post-fixing in reduced osmium as you state is my other suggestion, but see if ethanolic UA gives you what you need before re-embedding more samples. Also see if the two techniques combined (reduced osmium, ethanolic UA stain) give you even better results! Be sure to post your findings. Walter Bobrowski <walter.bobrowski@pfizer.com> 02 May 2007

It is possible that you are losing your cellular membranes during the dehydration steps. First, en bloc stain with saturated uranyl acetate in water and then go through the dehydration steps quickly, about 2 minutes for each step. Begin with 50 % ethanol and end with only one change of 100 % ethanol. If you are infiltrating with ethanol: araldite, keep the steps with high ethanol content short, too, about a 30 minute maximum. Dotty Sorenson <dsoren@umich.edu> 02 May 2007

Along this same line of thought, if you have access to a variable-wattage laboratory microwave suitable for histo- or EM processing, you can drastically shorten your dehydration solvent exposure times to seconds, rather than half an hour or more. Processing times for all steps are greatly reduced by using microwaves. It is possible to go from fresh sample to polymerized blocks in 4-5 hours, or sometimes less, with often superior results compared to conventional processing. Extraction of sample components is minimized by the short exposures to the various reagents, especially in dehydration steps. You can find our "generic" microwave protocol

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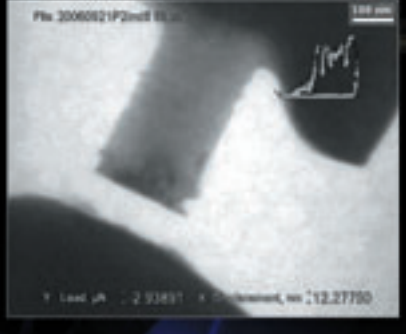

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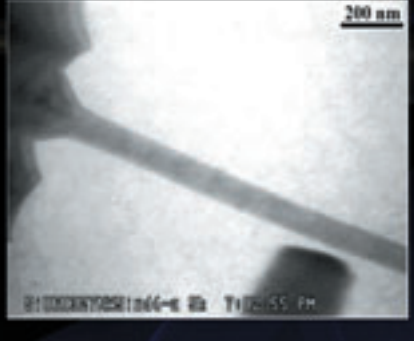

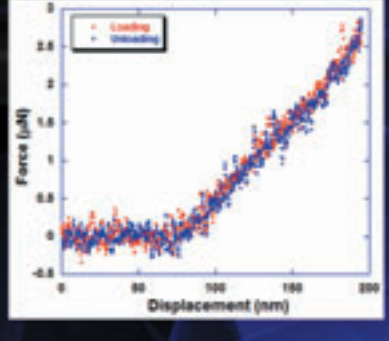

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at_http://www.emc.missouri.edu/Pdfs/General%202-ME%20Microwave%20Processing %20Protocol.pdf. Like most everything else in EM work, it gets modified all the time, but it's a good starting point. Randy Tindall <tindallr@missouri.edu> 02 May 2007

I am sure someone else can elaborate more than I, but Hyatt states that phosphotungstic acid after glutaraldehyde fixation in an aqueous acidic medium intensely stains the mitochondrial matrix, cisternae of endoplasmic reticulum, and others. This is from "Principles and Techniques of Electron Microscopy", 4th edition, page 313. Rhonda Allen <rra@stowers-institute.org> 03 May 2007

For OsO_4 to be reduced to OsO_2 (as happens during fixation) requires the presence of hydrogen ions and an electron donor. Electrons can be supplied from breaking up the double bonds in fatty acid alkyl chains of unsaturated (membrane) lipids. The reaction is reversible, so re-oxidation is theoretically possible, but only if a stronger oxidizer with a redox potential more positive than the one associated with the OsO_4/OsO_2 redox couple (Eo =1.02 Volts) is present. And even then, re-oxidation will only occur under suitable circumstances! I found Carol's comment that hexylene glycol preserves the osmium contrast very interesting. What could it be in ethanol that might re-oxidize the osmium? Peroxides? Chlorine? Oxygen in statu nascendi? OsO_4 as such is a pretty strong oxidizing agent already. Very puzzling. I hope I am not making any serious mistakes here, I am not a chemist (I wish!), just trying to understand how it works, but it seems a slightly acidic environment would be good to promote the fixation as well as to preserve the OsO_2 in the tissue after fixation. Could it be that the ethanol is alkaline? Even though ethanol is still somewhat polar, it may not take much in a non-aqueous environment. I am sure there will be proper chemists and physicists subscribing to this great listserver. Maybe they would be willing to look into our mostly empirically established procedures; we might be able to make a big move forward. Jan Leunissen <leunissen@aurion.nl> 04 May 2007

I just thought I should mention something that I think I read years ago in one of M.A. Hayat's books. He mentions that cacodylate and some other buffers when incorporated into a fixative produce higher contrast because they are more extractive, whereas phosphate buffers probably preserve cell contents better but at the expense of contrast. I just thought that I should add this because you mention Sorenson's buffer in the fixative. I'm sure that many of the other points have an effect but just wondered if the buffer could be contributing to your problems as well. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 10 May 2007

I am so glad to see the discussion on the contrast issue continues as I have been hearing more and more reports on this problem and I myself have been experiencing it too. Many hypotheses have been suggested on the causes of the problem but it seems that there were always evidences supporting different arguments. Practically, besides using short dehydration and infiltration times for monolayer cells, I now have to use potassium ferrocyanide with OsO_4 in order to get good contrast (even for bulk tissue). When I find out the contrast is low after sections are being cut, I re-counterstain sections with 15% uranyl acetate in methanol (it can give a muddy appearance if it is over done). In addition, I make sure to use a relatively fresh OsO_4 stock solution. I have been working in EM core facilities for a long time and have dealt with all kinds of samples. Hong Yi <hyi@emory.edu> 10 May 2007

SPECIMEN PREPARATION - plant fixation

At the request of an investigator who was interested in plant microtubules, we fixed Arabidopsis hypocotyles in: 2.5% formaldehyde, 2% glutaraldehyde, in PEMT (100mM PIPES-KOH, pH 6.9, 5mM EGTA, 2 mM MgCl2, 0.05% Triton X-100) for 1h at room temperature. This was followed by standard 2% OsO4, ethanol dehydration and embedding in Spurr's resin. The results were less than pleasing. The membranes seemed soft with little crisp clarity anywhere. In contrast, we fixed maize leaves using: 2.5% glutaraldehyde + 2% paraformaldehyde in 0.1 M cacodylate, pH 6.8, as the primary fix with the rest the same as above and got great membranes, sharp chloroplast granae stacks, etc. Only real difference was the buffer. Have any of you used a similar PIPES buffer and do you have any comments/ideas of why the one prep was good and the other not? Debby Sherman <dsherman@purdue.edu> 08 May 2007

The first thing that I saw was Triton X-100! I don't imagine that including a surfactant with your primary fixative would be any good for the membranes. I have used straight PIPES (without the additives) in plant tissues with good results (but not better than with cacodylate or phosphate buffers). Kim Rensing <krensing@ucalgary.ca> 08 May 2007

SPECIMEN PREPARATION - cacodylate vs. phosphate buffer

I have a faculty member who wants to do a perfusion fix of brain (it's a rat or mouse I forget which). Normally I avoid using cacodylate buffer because of the arsenic. However, in the dim recesses of my memory I seem to recall that phosphate buffer is very prone to precipitate formation in brain tissue. Now I do not know if I am remembering this correctly. So, what are your opinions? Would it be better to use a cacodylate buffer or should I stick with the safer less toxic phosphate buffer? Tom Bargar tbargar@unmc.edu 23 May 2007

I have never used it for perfusion fixation but HEPES buffer works well in my standard fixes with 2% paraformaldehyde or 2% paraformaldehyde + 2.5% glutaraldehyde. Alternatively, PIPES is used by other researchers with success. I see no argument for retaining use of cacodylate. Tom Phillips <phillipst@missouri.edu> 23 May 2007

SPECIMEN PREPARATION - myelin osmication

I have an issue regarding perfusion fixed (glutaraldehyde and paraformaldehyde in cacodylate buffer) adult rat spinal cord. I have cut cord cross sections approx. 1-1.5 mm. thick, which were osmicated in a 1.0% OsO4/cacodylate buffer for 1.5 hrs. When I cut into the middle of the tissue block it was completely white — the osmium did not penetrate beyond the surface of the tissue. I know that the heavily myelinated spinal cord can be a barrier to complete diffusion of osmium post fixation. These blocks will be sectioned for light microscopy stained with Toluidine Blue to access myelin fiber numbers, then potentially TEM examination. Question: I have researched the literature and one paper suggested using 2.0% osmium and leaving the spinal cord blocks in overnight. Another suggested using potassium ferrocyanide with the osmium. One of our university EM experienced researchers suggested warming the osmium to 37°C. Karen Bentley <Karen_Bentley@URMC.Rochester.edu> 24 May 2007

https://doi.org/10.1017/S1551929500055796 Published online by Cambridge University Press

Is it possible for you to cut thinner cross-sections? I am working with a project that hands over to me fixed (similar fix to yours) rat spinal and ganglia sections around 40 microns. They are fully osmicated, black all the way through. I think they use a Vibratome to cut those sections. Gib Ahlstrand <ahlst007@umn.edu> 24 May 2007

Osmium will penetrate 1 mm maximum but if both surfaces of a cross section of spinal cord are exposed you 'should' be OK with 2 exposed surfaces. Are the sections really 1-1.5 mm thick? Are you agitating the tissue? Is the osmium reasonably fresh? I do not see how 2% osmium will solve the problem and ferrocyanide will not help if the osmium does not arrive at the 'target'. Warming the osmium might help but I do not see it making a huge difference. I suggest fresh osmium, longer times in osmium, frequent (continuous?) agitation. If that fails, try using a Vibratome to cut 0.5 -0.8 mm sections. If you are sectioning for TEM, are you really going to cut all the way through a 1.5 mm slice? If not, make the sections thinner. Geoff McAuliffe <mcauliff@umdnj.edu> 24 May 2007

In my experience with rat sciatic nerve, my pieces were several millimeters long (oriented for x-section view), but tended to be less wide than spinal cord, so I typically had great osmium penetration. However, I noted that several samples that were very wide tended to have poor osmium penetration throughout, particularly if I sectioned too deeply. Keeping that in mind, I suggest thinner tissue samples (no longer than 0.5mm), either by hand or Vibratome. Secondly, minimize tissue loss during your initial block face trimming and obtain your sections ASAP. Walter Bobrowski <walter.bobrowski@pfizer.com> 25 May 2007

SPECIMEN PREPARATION - alternative to uranyl acetate stain

I am not able to use uranyl acetate. I would like to stain some osmium-fixed tissue thin-sections for TEM; the procedure I am following has a UA/Sato's lead stain step for the sections. Does anyone know of a suitable alternative? Jessica Cervantes <cervantes@bendres.com> 13 Apr 2007

In 1964, I published a small note about the potential use of Indium Trichloride as a stain during embedding (J de Microscopie, 3: pp575- 578). As I recall, it added some contrast, specifically to virus structures. It might be worth considering. Joel <jbs@temple.edu> 16 Apr 2007

You will get even more contrast if you use $KMnO_4$ followed by Sato's, as we have since 1964 (when Pb was was Pb citrate, not Sato's). In addition, you can get good contrast on thinner sections. Using tannic acid in the block stain, before OsO_4, or after but followed by uranyl acetate, adds to contrast, improves preservation. We have had excellent results. Some reports of using tannic as a section stain before Pb stain can also be found with Google's help. I've never tried it yet. $KMnO_4$ section stain won't work if NMA is in your Epon mix. Mike Reedy <mike.reedy@cellbio.duke.edu> 18 Apr 2007

Don't know about $KMnO_4$ staining compatibility with LR White; please let me know. The easy test is to put a blank block or chunk or flake of cured LR White in 1-2% $KMnO_4$ and soak it for an hour or a day to see if resin surface turns light brown, dark brown, or black and charred looking. No reaction is good news for section staining. The more stringent assay for reaction is to put the test tube of $KMnO_4$ solution with the cured embedding resin in a beaker of water and then heat the water to boiling for an hour or so, cool it off and examine the resin surface for such changes. Araldite 506 (lowest viscosity one I know of) and other Araldites used for embedding are remarkable resistant to surface discoloration when this is done. Epon DDSA (no MNA; fiddle the mix until it is hard enough to please you, and forget the epoxy:anhydride ratio lore) turns moderately browner, as I recall, but is still acceptable for section staining, with some tendency to be more granular and maybe show some hard-to-eliminate nano-pepper stain deposit in contrast to Araldite. LR White is an acrylic. EMS says LR White is a polar monomer polyhydroxylated acromatic acrylic resin. It can be cured by heat or by UV light. Sections of polymerized LR White resin are hydrophilic. I think Lawn's 1960 Journal of Cell Biology paper introducing $KMnO_4$ section staining used sections of methacrylate, the mix of methyl and butyl methacrylate we all used in the late 1950s before epoxies, especially Epon, turned up and became dominant. Tannic acid is a fixative and a mordant, and magically capable of superior structure preservation and assuring really strong uniform staining in our hands-- see the evidence of 13Å preservation in fiber x-ray diffraction from fixed-embedded fibers in Sader et al (2007) J Struct Biol (Articles In Press on line), and look at EMs reprints for evidence of the good morphology. The latter is a wondrous gift of tannic acid I had no idea of until I learned it from David Begg et al, J. Cell Biol. 79:846-852, an all-time key paper in my book of methodological turning points.. The other key was the observation by Hirose and Wakabayashi (J. Mol. Biol. 204:797-801) that tannic acid followed by OsO_4 or uranyl acetate give great morphological fixation without aldehydes. They used it for freeze-substitution, as we have, but we found it also worked very well as a general fix (we termed this TAURAC) for permeabilized cells in aqueous buffers so long as we excluded tannic acid blockers like PVP, Triton X-100, etc. Your uranyl acetate police might be interested in a demonstration of how completely tannins, esp. tannic acid, can convert a uranyl acetate solution into a flocculent brown precipitate at the bottom of a uranyl acetate-free supernatant (it looks uranyl acetate free! I made no measurements.). Tannic acid is cheap in non-EM grades; maybe they'd accept precipitation as a way of rendering it safely bio-inactive. From the information online at EMS is their datasheet/22400, I've estimated that the 10,400 counts/sec in depleted uranium is reduced to about -2 counts/sec in a very small bundle of muscle fibers containing 1 µg of protein and binding maybe 5 µg of uranyl acetate. But it still requires health police and special containment if such a specimen is to be transported into or within the DOE facility at Argonne National Laboratory. The inconvenience has so far discouraged me from pursuing some experiments that would require such a specimen, but one day I will do it, legally and according to their regulations. I doubt a Geiger counter could detect any rise above background in the presence of that small an amount of uranyl acetate. Mike Reddy <mike.reedy@cellbio.duke.edu> 14 Apr 2007

Has any one suggested p-phenylenediamine? Numerous references to using p-phenylenediamine but one to start with is "The use of p-phenylenediamine in the block to enhance osmium staining for electron microscopy" Stain Technology 47(5):239-243. Allan Mitchell <allan.mitchell@stonebow.otago.ac.nz> 18 Apr 2007

Allan Mitchell suggested p-phenylenediamine as an osmium

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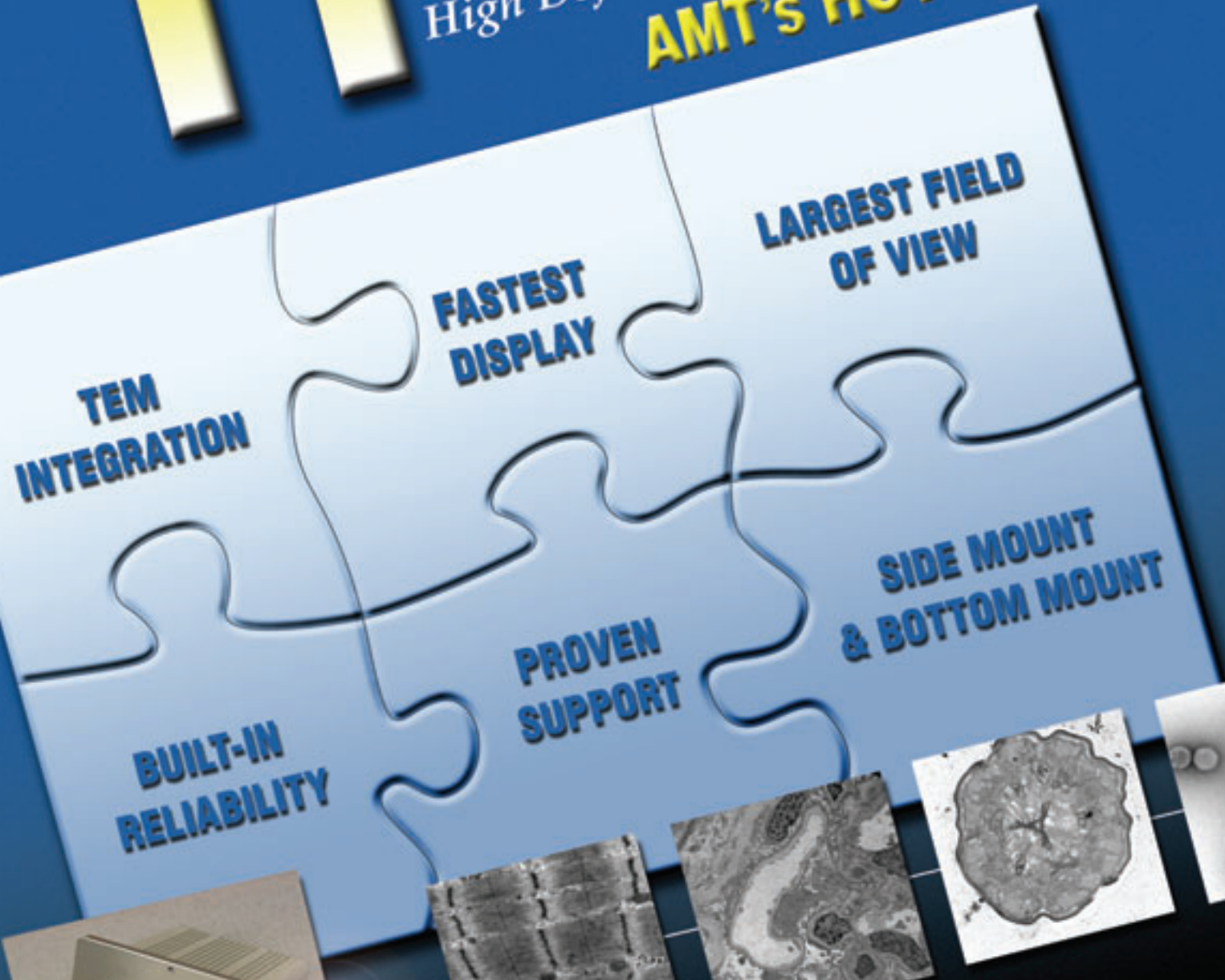

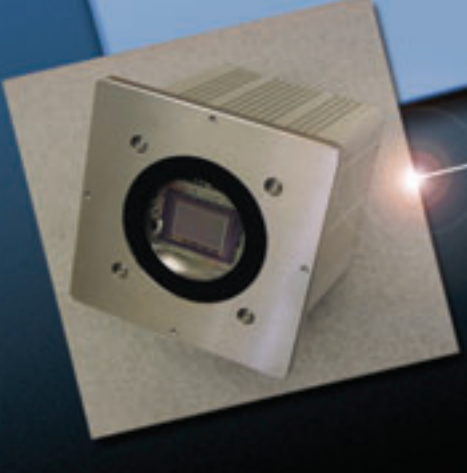

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'enhancer' and I agree. I have used p-phenylenediamine in 70% ethanol during dehydration for years; it chemically reduces osmium bound to the tissue and increases contrast. The only drawback is that tissue so treated is difficult to stain with the usual toluidine blue + borax solution. Geoff McAuliff <mcauliff@umdnj.edu> 18 Apr 2007

We get nice contrast with 1% potassium permanganate (aq) followed by lead citrate. It is good for membranes. In the past we have made it up in 0.1 M phosphate buffer at pH below 6.5 to avoid precipitates. Dave Patton <david.patton@uwe.ac.uk> 19 Apr 2007

What embedding plastics have you used? My lab has always used straight aqueous $KMnO_4$, following with dilute Pal's bleach to get rid of seemingly inevitable dusting of fine MnO_2 (I presume) precipitate, as suggested by Robertson et al. Does pH control work reliably? Where did you get the idea? I learned by experiment in the 1960s that over-long staining with $KMnO_4$ can "digest" the stained ultrastructure right out of the epoxy section; and Pal's bleach can extract the stain, so timing has to be reasonably limited with both steps to get the benefits without the losses. Mike Reddy <mike.reedy@cellbio.duke.edu> 19 Apr 2007

SPECIMEN PREPARATION - lead citrate and block staining

I've just read a paper where the author talks about block staining with uranyl acetate and lead citrate. Uranyl acetate yes, but Pb citrate? Has anyone heard of this? Does it work? The pictures in the paper were very nice; good contrast and detail. With the recent talk about general lack of contrast in specimens these days (I quite agree on that; I find contrast poorer now than in the past), could this be a new method? Diana van Driel <dianavd@eye.usyd.edu.au> 25 May 2007

I use Pb in block and not on the grid. Works great. No lead pepper. See my article in Microscopy Today, January 2007. David Elliott <elliott@arizona.edu> 25 May 2007

Geoff McA asked for the reference to the paper I mentioned. It's Peters S et al; American Journal of Ophthalmology (2007) 143:995-1002; Ultrastructural findings in the primate eye after intravitreal injection of Bevacizumab. All it says is "postfixed with 1% OsO_4 at room temperature in 0.1 M cacodylate buffer (pH 7.4) for three hours, bloc-stained with uranyl acetate and lead citrate, and embedded in Epon after dehydration in a graded series of acetones" I've e-mailed asking for more details. The author replied that PbC was not used as a block stain. It was an English usage problem - the author is German - she actually used the Pb citrate in the usual way as a section stain. Diana van Driel <dianavd@eye.usyd.edu.au> 06 Jun 2007

Dr. Peters gives me permission today by email to quote on the List Server her clarification/correction: "Our method is as follows: We use uranyl acetate in 70% ethanol as a block stain ..... then [after] the tissue is embedded in Epon and polymerized. We use the lead citrate only for the ready prepared sections. "I am sorry if my phrasing [turned out to] be kind of misleading." I plan continued exploration of both lead salts. Use as a block stain in organic solvent after uranyl acetate especially interests me for freeze-substitution. So far, I find both are insoluble or nearly so in 100% acetone, so the ethanol-containing vehicle in Elliott's procedure seems necessary. Does anyone know or remember if the extreme alkalinity of aqueous lead citrate section stains is essential for releasing the Pb from the strongly sequestering citrate? I'm not sure that pH 11 can be approached in pure organic solvent, but why not try....? I would guess that prolonging the exposure of tissue blocks to organic solvent for extended block-staining is NOT likely to cause additional shrinkage or extraction of cells. Because I think the binding of metals tends to stabilize the components - not completely against shrinkage. However; I find that freeze-substitution in acetone-TA followed by acetone uranyl acetate fails to prevent variable amounts of shrinkage, 5-12%, of the myofilament lattice spacing in striated muscle. I'd like someday to see the process monitored by x-ray cryo-diffraction to identify the stage where this occurs and seek ways to prevent or minimize the shrinkage. Mike Reedy <mike.reedy@cellbio.duke.edu> 05 Jun 2007

SPECIMEN PREPARATION - dehydration of molds

I am trying to figure out the best way to dehydrate and dry two types of mold, Rhizopus and A. Niger. I am going to osmium vapor fix them overnight but I would like to know if any of you have any suggestions for the dehydration and drying procedures. I am worried since the samples are so delicate. I am going to be viewing both samples using the SEM. Adena Rollins <arollins@hotmail.com> 27 Apr 2007

If you are quick, you do not need to do any fixation. I did this with Zeiss Supra 55VP at 200V EHT. This was with the in-lens detector. No coating. This has to be done fast since the clusters will implode when subjected to high vacuum. VP is not all that great. Do HV and quickly. Gary Gaugler <gary@gaugler.com> 27 Apr 2007

One technique I have used successfully in the past is fixing with osmium vapor, dehydrating using vapor diffusion dehydration and then critical point drying. This can preserve the conidial structure of Aspergillus very well. The original reference is: EJ King and MF Brown 1983 A technique for preserving aerial fungal structures for scanning electron microscopy. Canadian Journal of Microbiology 29:653-658 Ian IHallett@hortresearch.co.nz> 27 Apr 2007

SPECIMEN PREPARATION – ruthenium oxide

My question is about TEM and staining with RuO4. I am using this method to enhance the contrast of my sample and it is working properly, but I am trying to find some bibliography or some explanation about the mechanism, it is not very well described in any of the papers and book I've seen. I know it is an oxidation process but how it is attaching to the molecule and more important to me, how it is modifying the size? Patrica Forcen <pforcen@unizar.es> 18 May 2007

The best reference I know is Trent, J.S. et al, Macromolecules, 1983, 16, 589. Trent deals with a variety of issues including specificity of staining by RuO_4 and OsO_4 and the chemistry of the reactions. I suggest you also look in the Polymer Microscopy, by Sawyer and Grubbs, 2nd ed. for other references. Gary Brown <gary.m.brown@exxonmobil.com> 18 May 2007

The key to understanding the mechanism is that the structure of ruthenium tetroxide is the same as that of osmium tetroxide,

a central heavy metal bound by double bonds to 4 oxygens. The reaction is the same as for osmium when it interacts with organic material - breaking of one of the double bonds by the target and then cross-linking of the target with the RuO_4. Contrast is the result of added density of the heavy metal to the target. Hayat is relatively quiet about the chemical reaction in his Fixation for Electron Microscopy. I seem to have my copy of his Positive Staining for Electron Microscopy at home so I don't know if he even covers it as a stain, my memory is that he doesn't. It is also not covered in Lewis and Knight's Staining Methods for Sectioned Material. Paul R. Hazelton <paul_hazelton@umanitoba.ca> 18 May 2007

Like Paul, I'm working from memory. However, I recall (hopefully accurate) information on mechanisms from Sawyer and Grubbs or Trent. I believe that the mechanisms for OsO_4 and RuO_4 oxidation of polyolefins are different. OsO_4 adds across C=C bonds and does not react with C-C bonds at all. RuO_4, on the other hand, scissions C-C or C=C bonds to produce oxygenated chain ends. Gary Brown <gary.m.brown@exxonmobil.com> 18 May 2007

SPECIMEN PREPARATION - bacterial flagella

Does anyone have any tips for preparing and imaging bacterial flagella at reasonably high resolutions? I need to examine the flagella and measure potential differences in their width, so I'd like to image them at around 100-200kx. The flagella are about 25nm wide. So far, I've been making cell-free flagella preps and adsorbing this to carbon-coated copper or nickel grids, and then staining with PTA. They don't look too bad, but I'd like to know if there would be better methods for this kind of thing. Scott Coutts <scott.coutts@med.monash.edu.au> 17 May 2007

Carbon-coated (copper) grids: yes if you just want to know their width, negative staining appears appropriate and a final magnification of 30,000 to 50,.000 x (on film, on CCD-camera) should be sufficient. Stain: PTA might be ok, but you may also try other stains in parallel samples, like uranyl acetate or uranyl formate. You also may add some sugar derivatives (glucose, trehalose) for preventing minimizing the collapse of the flagella upon air-drying. Ultimately, you may try cryo-TEM of unstained samples; this will give you the samples with highest quality. Reinhard Rachel <reinhard.rachel@biologie.uni-regensburg.de> 17 May 2007

Hit Pubmed and look up the pioneering experts' papers, Namba, DeRosier especially. I've not read them to see how they compensate for flattening. But in my field of interest, muscle myosin filaments, the classic way to minimize flattening is tannic acid followed by uranyl acetate for negative stain, as in (1986) Stewart, M. and Kensler, R. W. Arrangement of myosin heads in relaxed thick filaments from frog skeletal muscle. J. Mol. Biol. 192:831-851. I think a still earlier 1985 paper about scorpion filaments may have introduced the method. I think Kensler has used that method ever since, certainly in Journal of Structural Biology 137, 154-163 (2002) where it is described in detail. I'll send you that paper off-list. Mike Reedy <mike.reedy@cellbio.duke.edu> 17 May 2007

SPECIMEN PREPARATION - bacterial samples for SEM

I have obtained a sample of freeze-dried bacteria, and would like to prepare it for the SEM. Is there something more than just double-stick carbon disk on a stub and sputter coating if it's already freeze dried? -- Justin Kraft <kraftpiano@gmail.com> 15 May 2007

My limited experience with preparing bacteria for SEM, I

can say that I would NOT recommend to just apply 'freeze-dried bacteria' to a double-stick carbon disk, and then sputter-coat the stuff. These freeze-dried bacteria are most likely completely collapsed and therefore will look 'collapsed' and 'dead'. I would suggest to recultivate the cells in a suitable culture medium, then either fix them chemically, apply them to a filter (like 'Nuclepore'), dehydrate, followed by critical-point drying and sputter coating. Or: apply them to a filter, fix them physically (plunge-freezing), then dehydrate at low temperature by freeze-drying at -80 C, then sputter-coating at low temperature or even rotary-shadowing at low-temperature and then SEM. Depending on the type of bacteria, many variations of these protocols are possible and might be necessary. Reinhard Rachel <reinhard.rachel@biologie.uni-regensburg.de> 15 May 2007

SPECIMEN PREPARATION - cationic dyes for bacterial visualization in SEM

Recently I started working on visualization of bacteria on various biocidal surfaces. I have a new question regarding implementation of cationic dyes for SEM bacterial visualization. Considering that I am primarily looking for changes (sometimes very subtle) in bacterial appearance due to the biocide action I am concerned that some of the changes could be introduced by fixation/processing artifacts. Will the addition of cationic dye to glutaraldehyde/osmium tetroxide protocol help me to preserve fine structures? Which dye would be best and in which concentration? Would it make difference for gram-positive as much as gram-negative strains? Albina Mikhaylova <amich@ufl.edu> 23 Apr 2007

Both ruthenium red and Alcian blue can be used. In addition cetyl pyridimium chloride is also a choice for preserving negatively charged mucopolysaccharides that are on the surface of some cells. The tendency is to overly decorate these surface coats and obscure real detail. These reagents do nothing to preserve the actual cell or the cells wall. I also think that SEM is not the tool for seeing any subtle effects. Work I had done some years ago required thin section TEM and even then only catastrophic changes were evident i.e. lysis or cytoplasmic partitioning. Changes in membrane architecture might require freeze fracture EM. To minimize fixation artifacts for SEM you might try rapid freezing followed by freeze drying; freeze substitution fixation followed by critical point drying or cryo-SEM of fully hydrated cells. In all three of these techniques very rapid freezing is required in order to prevent ice crystal formation that would cause artifact in and of itself. There is a book by Aldrich and Taylor that discusses in more detail some of the things that I have mentioned. I think the title is "Ultrastructure Techniques for Microorganisms". It is old, but many of the techniques are still in use today. Greg Erdos <gwe@ufl.edu> 23 Apr 2007

SPECIMEN PREPARATION - progressive lowering of temperature technique

I am having some sectioning difficulties with cross sections of cultured cells grown on Thermanox coverslips. The monolayer of cells grown on the coverslips have been processed by the progressive lowering of temperature technique (in an AFS), using ethanol as the dehydration solvent, and then followed by embedding in Lowicryl HM20. The problem I am having is that the Thermanox coverslips are separating from the Lowicryl HM20 resin block making cross-sectional ultrathin sectioning (and semi-thin for that matter) that includes the coverslip difficult. The separation is easily seen through the binocular head of the ultramicrotome even before I start trimming the block down. The resin simply hasn't 'bonded' to the coverslip. (I am able to remove the coverslip from the block and then get good ultrathin sections of the cells embedded in the resin but we would prefer to keep the coverslip in place if possible). A parallel run of cells grown on Thermanox coverslips, processed at room temperature and embedded in conventional epoxy resin are working fine. I can easily get good ultrathin cross sections from these which includes the coverslip and cells. My question is: Has anyone had success with ultrathin cross sections of Thermanox coverslips and cells embedded in Lowicryl resins? Allan Mitchell <ell@stonebow.otago.ac.nz> 18 May 2007

We have had the same problem in that only the epoxy resins will bind to the Thermanox, and Aclar for that matter. Are you doing PLT and HM20 embedding for immunolabeling, or just to avoid room temp dehydration? I know some people who have had success immunolabeling after etching the epoxy resin away (just an idea). We have had the most success growing cells on a Transwell Plate (sold through Corning here in Canada) that has a porous polyester membrane on the bottom. The HM20 resin is able to penetrate through the pores and will hold everything together during sectioning allowing for decent thin sectioning. Expect some wrinkles and getting a perfect flat cross section is difficult because the membrane is pretty flexible, but it works. Garnet Martens <gmartens@interchange.ubc.ca> 18 May 2007

SPECIMEN PREPARATION - metal shadow casting of DNA

We are currently trying to rotary shadow cast DNA but are having problems obtaining consistent results with the metal shadowing. We are using an 80:20 platinum palladium wire purchased from EMS and a Denton Bench Top Turbo III sputter coater. The platinum palladium wire is wrapped around a 2-3 cm portion of a hairpin loop formed with tungsten wire. When the filament power is increased, the platinum palladium wire will either heat up without melting and evaporating or else the tungsten wire breaks without appreciable metal shadowing. I was wondering if anyone had any experience with this technique and could offer suggestions about how to obtain more consistent results. Shannon Modla <modla@dbi.udel.edu> 01 Jun 2007

I don't know the particulars of operation of the evaporator unit and my knowledge is for pure gold or platinum (some metals will alloy with the tungsten and cause problems - maybe someone else knows). Platinum MP = 1772 C Tungsten MP = 3410 C. If the Pt wire is not melting, then the tungsten temperature is too low. If you advance the filament heating slowly (a few seconds for work, but slower in testing until you know the behavior) you should first see the Pt:Pd wire melt and flow to the bottom of the hairpin (the point of the V is down) and the droplet will evaporate at a slightly higher temperature. Actually, you only need to put the wire near the tip of the V. Just remember to heat the filament over a few seconds, not instantly. Tungsten has a positive temperature coefficient and the filament resistance is initially low and a large surge will occur if full voltage is applied directly - it can cause the evaporant wire to spit or the filament to burn out; but you shouldn't need to be

that close to the tungsten melting point. You don't specify distances but it sounds like you are using a lot of wire. You probably only need 10-20 Å. 0.5 cm of 8 mil wire at 10 cm distance that will give ~13 Å (at normal incidence, or the sides of DNA so disposed....) Å = (40.3 * diameter^2 * Length) distance^2 diameter in "mils" (0.001"), 8 mils is a standard size, 0.008" diameter length of wire evaporant, distance to sample in cm. I have an Excel spreadsheet with a "calculator" for this formula that I can send if you want. Dale Callaham <dac@research.umass.edu> 01 Jun 2007

I have always thought that the best way to "shadow" was to use the "Pt/C sessile drop" method. I learned about it many years ago in graduate school and don't know who used it originally. But one gets a finer grain if Pt/C is evaporated simultaneously than if Pt alone is evaporated. The way to do this is to take a carbon rod that has been sharpened down to a "neck", wrap about the "neck" not more than 30-40 mm of 8 mil Pt wire, put the bell jar onto the vacuum evaporator but don't pump down. Slowly increase the current through the carbon rods to the point where, just beyond cherry red, the Pt melts and because of surface tension effects, and the fact that liquid Pt does not want to wet carbon, it "beads up", just as water does on a freshly waxed car. Once this happens, turn off the current, and when it cools down, rotate the bead so that it is facing the surface to be shadowed. Then the bell jar is put in place, the chamber pumped down, and the carbon rod is evaporated but what really comes off is a combination of Pt and C. Note: The optimum amount of wire to be used depends on a) distance to the substrate to be coated and b) shadowing angle. Since Pt wants to alloy readily with W, this approach avoids all those other issues as well. Charles Garber <cgarber@2spi.com> 04 Jun 2007

SPECIMEN PREPARATION – cross-sectioning packaging film

I am a relative newcomer to the microscopy/histology field and need some advice on sample preparation. I am trying to section a multilayer packaging film (e.g. polyester, aluminum foil, polyethylene lamination) and am having trouble keeping the sample flat, so I can get a good reading on individual layer thicknesses. I am using a cryostat microtome for sample preparation. Is there a simple technique I can employ, or a type of embedding compound that works better for industrial applications? R. Jefferson Babbitt <jbabbitt@fresco.com> 20 Apr 2007

I would try freezing the sample and using a single bend fracture procedure as set out below. We have used this technique on polythene freezer bags and many other materials for SEM but the technique is equally applicable to LM. "Over the many years that clients and I have been investigating the cross sections of materials the best method is to fracture the material. The SEM is very clever in that it sees a cut surface and tells us "this is a cross section cut with a sharp scalpel blade" or "this is a cross section cut with a blunt scalpel blade" etc. Preparation method A 1. Cut down the material to 1cm by 3cms place it into liquid nitrogen until it stops bubbling. 2. Remove the material and crack it using either heavy duty tweezers or fine pliers. If you are unable to crack the material and are forced to flex it in order for it to crack this is not good enough! In the latter case reduce or neck the material, even a 0.5mm long crack could provide a great deal of detail in the SEM. 3. When the pieces have dried out (condensation) they may both be observed by LM and SEM. Fibers that will not fracture by the above method could

be fractured by one of two other methods. Method B 1. Insert the material in a small diameter tube (thin drinking straws are ideal). Cut the straw down to about 3 cm tall. Block one end with wax, modeling clay or similar material. 2. Using a syringe force water into the straw and block the end as above. 3. Drop the straw into liquid nitrogen then follow method A part 2 above. 4. When the pieces have dried out (condensation) they may be observed by both LM and SEM Method C 1. Drill 2mm to 3mm holes in a pair of stubs. Infiltrate the holes with a water soluble carbon solution and push a bundle of fibers through the carbon solution. 3. When dry follow method A part 2 except use a blade to initiate the crack 4. When the pieces have dried out (condensation) they may be observed by both LM and SEM Method C has been used with materials like freezer bags that were failing. In this case the material was spun into a small spiral and then infiltrated with the carbon solution." The above data was taken from our "hints and tips" web page. Steve Chapman <protrain@emcourses.com> 20 Apr 2007

SPECIMEN PREPARATION - aluminum evaporation

I have been asked what the voltage and current conditions are for evaporation of aluminum in a tungsten basket in a vacuum evaporator. Is this sort of information available in a reference? I don't have much experience with metal evaporation, so any advice would be appreciated. Kim Rensing <krensing@ucalgary.ca> 11 Jun 2007

Voltage and current requirements vary depending upon the volume of aluminum you are evaporating. We coat substrates in the Ladd evaporator by starting out with a very low voltage and adjusting it higher till the aluminum starts to evaporate. If you wish you can call Mike Bouchard at Ladd - 1-800-451-3406 to discuss particulars. He has many years of experience in the manufacture of the Ladd evaporator and substrate coating. John Arnott<jd@laddresearch.com> 11 Jun 2007

There is no set voltage/current to evaporate aluminum. A variable power source is used to achieve this. A current is gradually increased until aluminum melts and then a little bit more increase starts the evaporation. We achieved good results using a tungsten wire with v-shaped bend on which a small v-shaped piece of Al wire was hung. Alexander Titkov <alex.titkov@millenniumchem.com> 12 Jun 2007

SPECIMEN PREPARTION – sputter coating

I have a Polaron SC-502 sputter coater in the SEM lab. One of the Engineering faculty wants to coat samples with chromium, titanium and aluminum for an application other than SEM. Assuming I can get targets that fit the sputter coater, are there other issues that would prevent using these other metals? Dave Wilbur <david.wilbur@tufts.edu> 25 May 2007

We use our Emitech coater to coat with a variety of metals, including chromium, platinum, tantalum, and others. You will have to check on sputtering currents and times and how they relate to coating thickness for whatever metal you're using, but I'm not personally aware of any reason you can't use the Polaron for a variety of metals. My real reason for replying is to get one of the best-kept secrets in the World of Sputtering out there. You can get custom-made targets of virtually any metal from Refining Systems, Inc. in Las Vegas, NV. These targets can be made to your specified diameters, shapes and thicknesses, usually at substantially less cost than targets from coater manufacturers. The company is run by Mr. Abe Dayani, who has always answered the phone himself when I've called. Website is at http://www.refiningsystems.com/, phone is 702-368-0579. I have heard that the purity of the metals used by the OEMs may be higher (like 99.998 vs. 99.99, for example), but I can say that we have never had any problem with the coatings from the RFI targets. It may be a factor for super-critical applications in engineering, so you may want to consider this. I have no connection with RFI other than as a satisfied customer. Randy Tindall <tindallr@missouri.edu> 25 May 2007

I am not familiar with the specific sputter coater you are using. So here are some questions. Do you know if it is a straight DC sputter coater or magnitron sputter coater? What is the pumping system, just a mechanical pump? Most sputter coaters for SEM sample prep only use a mechanical pump for vacuum generation. This means you are only going to about 10-3 torr of vacuum before backfilling and then coating. For most thin film depositions, this is quite poor vacuum. Most thin film sputter coaters will go to somewhere around 10-6 to 10-8 torr before backfilling for sputter deposition. They also usually have a way to clean the substrate prior to deposition, which your little sputter coater I imagine does not have. The amount of contamination you will have, both on the first coat and then when switching between targets will be much larger than usually considered acceptable for thin film deposition. All that being said, I don't see any reason you can't use your machine to deposit the materials you are mentioning, but I do question how useful the resulting layered structure will end up being. I would have to know a lot more about what the desired end product is to give you my thoughts on that. dj <dljones@bestweb.net> 25 May 2007

In fear if you have a conventional sputter coater - not the high vacuum versions that are designed for more difficult materials like chromium - you will not be able to coat other than with the simple materials - gold, platinum or gold-palladium. Basically the everyday SEM sputter coater does not have the power. Steve Chapman <protrain@emcourses.com> 25 May 2007

Let me add a little to this discussion. Even if the coater would have a high vacuum, there still are issues with these three materials. All of them have an oxide coating on the targets. Ti and Al are very thin. Cr takes longer to build a thicker oxide coating. You have to sputter the oxide away before you can use it to deposit the film. Ti is a getter material and so is Al to a lesser degree. What this means is that the thin film deposited will act as a pump to pump reactive species such as O2, H2O, H2, from the vacuum system. So you have to sputter for a significant time prior to actually laying the film down so that these species are pumped from the chamber and your film on your substrate does not act as a getter pump. Ti and Al will instantaneously oxidize when the sample is brought up to air, but it will be a very thin protective coat. This is important with respect to the properties and the thickness of your film. Cr will take longer to fully oxidize, but it will unless protected by something like our SampleSaver(TM) container. It would really help everything if your base pressure for your coater is at least in the 10-7 torr range or better. You might consider putting a top layer of a very thin Ti onto these other materials to act as a protective oxide layer. Scott

Walck <walck@southbaytech.com> 25 May 2007

Aluminum and titanium will deposit as alumina and titania unless you've got a turbopump or better. There is no easy solution. Even then, it will instantly oxidize upon exposure to air. If the SC-502 is a magnetron sputter unit, then the chrome may shunt the field. Jim Quinn <jquinn@www.matscieng.sunysb.edu> 25 May 2007

SPECIMEN PREPARATION – humidity levels

Can anybody give me an optimal percent relative humidity that is recommended for EM rooms? We recently had some failures with Formvar grids (we think) due to excessive room humidity when they were being made. I can bring in a de-humidifier, but what would be considered to be optimal? Teri Johnson <tjj@stowers-institute.org> 20 Apr 2007

If it is for Formvar that you wish to keep humidity low, you might consider casting your Formvar films within a glove bag flushed with dry nitrogen gas. We use such a system here in our Oregon lab with good success. Douglas Keene <drk@shcc.org> 20 Apr 2007

You need not only to be concerned about the Formvar, but you also do not want condensation on the column or electronics. Too low a humidity will also allow the generation of static electricity. About 40% RH is a very good value, and the maximum permissible depends on the difference in temperature between the room and the scope and is usually about 60%. Bill Tivol <tivol@caltech.edu> 20 Apr 2007

My opinion is that less than 40% humidity is ideal. Ted Dunn <drteddunne@yahoo.com> 22 Apr 2007

Just to clarify, this will be in a sample prep area only, the scope is in a different room. The ventilation in this room is "weird" (the best way I can describe it), most of the time it's not an issue. On occasion though, you can walk in to the room from the hallway and the atmospheric conditions are totally different. I'm thinking between 30 and 40% sounds about right. Teri Johnson <tjj@stowers-institute.org> 23 Apr 2007

Don't drop the humidity too low or static charge becomes, at least, annoying and, at worst, a problem. I would say 35% at the lowest. Ted Dunn <drteddunne@yahoo.com> 24 Apr 2007

IMAGING – digital compression

There is a thread currently discussing digital imaging and movies etc... Let me take a moment to remind you (or reinforce with others have said or implied) namely : if you save a file in any "lossy" compressed format your changing as well as loosing data. This must be reported if you use any of this processed data for any scientific analysis. The last time I checked there are some 60+ different image file formats. Lossless formats include (but are not limited to) the following: RAW, TIFF, BMP, PICT, PNG, PCX, EXR, SVG, TGA, JPEG2000 Lossy formats include: GIF, JPEG, MPEG, MOV, H263, Video On top of this there are also lossy and lossless COMPRESSION methods, which may be encoded within/upon some of these formats. So life can get very complicated , you might use a lossless method and then compress the data using a lossy algorithm. The Microscopy Society of America (MSA) has defined a policy on ethical digital imaging, which is available on line under Reference/Education section of their WWW site (http://www.microscopy.org) . This ethical

https://doi.org/10.1017/S1551929500055796 Published online by Cambridge University Press

position applies to all digital images, be they: still , time series or movies. While making movies from time series events etc.. is a valuable mechanism of looking through your data, (and I also do this frequently). Remember that digital imaging ethics requires that you store the original data using a procedure similar to that documented below. If you analyze your data you should refer back to the original uncompressed data, never to the compressed data. You also need to report these operation particularly if you down sample, compress, etc. your data. Consumer grade software does this routinely and likely does not tell you so. So be extremely careful when using some of this software for analysis of your scientific data! If you are recording directly to digital video, I would recommend that you record in RAW format to store that data and then after the fact convert your images to some alternative display. — Here is the MSA statement verbatum. — The MSA position on digital image processing has been approved as follows: "Ethical digital imaging requires that the original uncompressed image file be stored on archival media (e.g., CD-R) without any image manipulation or processing operation. All parameters of the production and acquisition of this file, as well as any subsequent processing steps, must be documented and reported to ensure reproducibility. Generally, acceptable (non-reportable) imaging operations include gamma correction, histogram stretching, and brightness and contrast adjustments. All other operations (such as Unsharp-Masking, Gaussian Blur, etc.) must be directly identified by the author as part of the experimental methodology. However, for diffraction data or any other image data that is used for subsequent quantification, all imaging operations must be reported." This policy was formulated by the Digital Image Processing & Ethics Group of the MSA Education Committee and was adopted as MSA policy at the Summer Council meeting August 2-3, 2003. Nestor Zaluzec <zaluzec@microscopy.com> 19 May 2007

Note that BMP used to be a lossless format, but since (I think) Windows 2000 a valid BMP file can in fact contain a JPEG compressed (i.e., lossy) image. I've yet to run into one of those "in the wild". In addition: GIF does not use lossy compression (but is never used for serious image processing). Regarding "RAW video": Suppose you are capturing full NTSC frames (there are different opinions on what the exact resolution is, but let's take 720x540 for the example) at 30 full frames per second (actually 29.97), in a raw format with three bytes per pixel this would take more than 32MB per second of footage. This would fill up a normal-sized hard disk quite rapidly. What is worse, up to a few years ago consumer grade hard disks could not even store data at this speed. Compression was not added to movie formats just to save space and costs; it was mainly because there simply wasn't any other way of doing real-time image acquisition otherwise. I don't know of any current (affordable) video cameras that can store their captured data in a raw format, but perhaps the state of the art has advanced more than I know. In addition, there is the problem that even if you pick a well-known image format and stay away from the "obscure" ones, you will still run into spotty support even on major software platforms. For example, I regularly get "complaints" from people claiming my TIFF exporter is broken because Word can't read the resulting TIFFs. Usually, that is because they have chosen 16-bit export, and Word only partially implements the (huge) TIFF specification and simply cannot read all valid TIFFs. Likewise, Windows Explorer won't show any thumbnails for those images, because it only handles 8 bit-per-channel TIFFs. The whole of "image postprocessing" is a bit of a gray area. If you use postprocessing to downsample an image you should report it, but if you select "binning 2" on your camera when doing the acquisition, you're in effect doing the same thing. Even worse, some consumer-grade cameras perform a host of image post processing in the camera itself to make their images look better. It's not uncommon to see an "artificial sharpening filter" being performed to get away with a cheaper lens system. I agree with reporting on the entire post-processing setup though, and with storing your original images in a lossless format, preferably TIFF, with sufficient bit depth (if you have a 10 bit TEM camera, don't store your images as BMP even if that format is listed as "lossless"). Even if some of your software cannot correctly handle these original files, there are plenty of (free) conversion libraries around (many open source, so they will stay around) which can convert it to any format you currently need. Sander Stoks <Sander.Stoks@fei.com> 19 May 2007

XTEM - surface densities of particles

Why is it not possible to obtain surface densities of particles from cross sectional samples (XTEM)? Is there any reference explaining this? Douglas da Silva <dls_douglasl@yahoo.com.br> 29 May 2007

There is no a priori reason why you can't measure densities of particles using cross section TEM. However, there are two things you need to be aware of: 1) The specimen is seen in transmission, so it is possible that you have two (or more) particles along the 'line of sight' of the electron beam. This can make it difficult to get an accurate count of the number of particles. 2) The specimen has a finite thickness (which might not be immediately apparent from a cross section image). To work out how many particles you have per unit area of the surface you need to know the specimen thickness. Both of these problems can be overcome by tilting the specimen so that the surface is not seen 'edge-on'. You can then work out the specimen thickness from trigonometry and the particles will not overlap. However the image may look more complicated and need more careful interpretation. If you want, you can also take a stereo pair and then you can see the true 3D structure. Richard Beanland <richard.beanland@bookham.com> 31 May 2007

SEM - smooth, low-Z substrates

We could use some suggestions/collective experience about solid substrates for doing SEM imaging of dispersed small particles. What we are imaging: silicate and oxide mineral grains and silicate glass particles down to the 0.1 micron size range (maybe smaller). Objective is to disperse the particles on a substrate and do total particle counting and automated measuring without "losing" the smallest particles from the data set. What we need: A substrate for dispersing the particles that would fit two requirements: 1) have a pretty low average Z (carbon would be ideal but I'm willing to consider other ideas), so that my particles will stand out strongly in BSE, and then 2) be as smooth as possible on the sub-micron scale. This last requirement is the tricky one because although there are lots of carbon substrates available commercially, so far we haven't found any that don't have sub-micron scratches and other roughness such that particles in the 0.1 micron size range don't get lost in the surface topography. Roy Christoffersen <rcsaic@sbcglobal.net> 14 May 2007

How about using a TEM carbon support film? Disperse your particles on the support film then mount the grid on a "Faraday cup" I made a TEM grid support by taking about a 1cm long piece of 1/4" graphite rod, drilling out the center 6-7mm deep, and using a 1/8" (3.1mm) end mill to make a small recess for the grid in the end of the rod. I used carbon dag to glue the rod upright on an SEM stub and presto... instant TEM grid holder with near zero background! Henk Colijn <colijn.1@osu.edu> 14 May 2007

Can you use a high-Z background rather than low? That should give you dark on light in place of light on dark... If so, you might want to investigate metal-glass braze foil. Typically it contains Si, Cr, Fe, Ni, or some combination (don't have the specs handy). Since it is a glassy metal, there is no grain structure to interfere with BSE imaging and the surface is relatively smooth. There are some undulations/waves in the surface formed when frozen from the molten state, but maybe not too much to interfere. Woody White<nwwhite@bwxt.com> 14 May 2007

There is a material called "glassy carbon", which is smooth, solid and can be polished to a mirror surface. It is very hard, but is pure carbon and so it makes a good substrate for BSE studies. Some of the EM catalogue companies used to supply it, they probably still do. I get my glassy carbon planchets (0.5 inch discs) from Canemco (www.canemco.com) and stick them onto my SEM stubs. Mary Fletcher <mager@interchange.ubc.ca> 14 May 2007

Have you tried carbon evaporated onto freshly-cleaved mica? The layer can be floated off and deposited onto lacy carbon so that there will be many areas several um across that have only the thin carbon substrate. The layer can be made to be only tens of nm thick, so it is very unlikely to have ~100 nm steps. Bill Tivol <tivol@caltech.edu> 14 May 2007

In my opinion, you should consider HOPG (highly ordered pyrolytic graphite), see URL for those who are not familiar with this unique material and its novel properties, it can be cleaved much like mica into very thin strippings, actually more easily than mica. This is done by pressing a piece of Scotch tape, either single sided or double sided onto the flat plate surface of the HOPG and then literally, stripping off a layer of HOPG which is left strongly adhering to the tape. This tape can then be mounted on a conventional SEM mount, perhaps with a double sided conductive carbon disc. Many additional strippings can be made from the resulting block until it is all consumed. The resulting stripping is highly (mirror) reflective, demonstrating the virtually zero porosity in the HOPG stripped layer. And depending on the grade of HOPG selected, there will be regions of varying area sizes of atomically smooth HOPG. You will not "lose" particles in the structure of the HOPG. There have been some suggestions by others, but even though we too offer glassy carbon, we recommend the HOPG over the glassy carbon for several reasons: a) Glassy carbon exhibits some porosity (depending on grade) on the scale of your fine particles that you don't want to lose and b) since one can get a number of individual strippings out of each HOPG plate, the cost per stripping (e.g. sample) is far less than the cost of a piece of glassy carbon. HOPG

is used widely in the field of AFM, one of the reasons being exactly the reason you gave: "...don't have sub-micron scratches and other roughness such that particles in the 0.1 micron size range don't get lost in the surface topography". Your specifications are consistent with the needs of AFM users looking at nano-sized particles (which cannot be cleaved). Disclaimer: SPI Supplies offers a complete line of HOPG and glassy carbon products and therefore we have a vested interest in seeing more of these items being used by researchers. See URL http://www.2spi.com/catalog/mounts/vitreous.html Chuck Garber <cgarber@2spi.com> 14 May 2007

Although I normally don't work with particles that small, most of our particle analysis is performed on carbon conductive tabs from Ted Pella Inc (catalog # 16084-4). There is some texture to the tabs, but usually the particles stand proud of the tab. With a backscatter image, the higher *Z* particles will stand out from the carbon. The problem with analyzing such a fine powder will be particle separation. We tend to sprinkle a small sample on the tab instead of pressing the tab into the powder, just to get a dispersion and provide separation between the particles,. Gerald Shulke <gas19@daimlerchrysler.com> 15 May 2007

Looks like Carbon is the idea material for your research. Have you tried to use natural graphite crystal -- it has beautiful layer structure and large flat surface area as well. Find a geologist and ask a piece of graphic crystal, stick it onto your SEM stub, then peel off first 3-10 layers to have fresh surface ready for use. Use sharp knife and peel from edge -- other people used tape to peel the top layers off. We used to make fresh "flaw-free" graphite surface for STM analysis. Xiang Yang <xyang@smu.ca> 15 May 2007

The artful use of a heat gun directed at a hot melt glue covered SEM stub will produce a lustrous surface on the low Z, low outgassing glue substrate. Perhaps while the glue is still warm and adhesive, you could sprinkle your sample onto it. Bart Cannon <cannonmp@comcast.net> 15 May 2007

SEM - image on glass

I have some patterned Cr structure(width: 100 μm, height: 60 nm) on glass. Then I spin 200 nm PMMA on the surface. Then I sputter 10nm Cr on the PMMA. I use a JSM 6400 SEM to look at the structure. But I can't see anything? What's the problem? Yitian <yitianp@ece.arizona.edu> 01 Jun 2007

Why did you "spin 200 nm PMMA on the surface?" As I understand, you just have a glassy flat surface of resin on your specimen, with no topography and nothing to observe. Vladimir Dusevich <dusevichv@umkc.edu> 01 Jun 2007

I was once given the task of looking at a glassy surface in the SEM. Just to rough in the focus was a challenge. I sprinkled some powder on the surface to provide a starting point for focus. Maybe this would help you get started. Otherwise, I agree with Vladimir... that PMMA - though optically transparent - is not electron transparent, and all you'll see is the Cr-sputter-coated polymer surface. Stu Smalinskas <smalinskas@yahoo.com> 01 Jun 2007

SEM - aluminosilicate and BSE

I want to detect aluminosilicate particles in the middle of an organic material. The particles are expected to be too small for light microscopy and too dilute for TEM. The solution would be to dry everything flat on an SEM stub and to find a way to differentiate organic particles from aluminosilicate particles. Our EDX doesn't want to start so I wondered if I could see something with BSE. X-from the atomic weight of the elements, Al and Si are not particularly heavy but they are of course very dense in the particles. Do you think it would be possible? Stephane Nizets <nizets2@yahoo.com> 18 Apr 2007

If you own a decent BSE detector, I'm sure that you will be able to see a contrast between the two types of particles. Average atomic number is well apart from each other. To make the observation easier, I would recommend using a low-Z SEM stub like graphite or a graphite plate on top of a standard holder. Then you will be able to see your silicates with a bright contrast with respect to the background and the other particles. Petra Wahlbring <petra.wahlbring@goodyear.com> 18 Apr 2007

That would be a good place to start. I have posted some images of inclusions in an organic goo on our web site (ftp://www.marl.iastate.edu/Interesting/Residue/). The mineral inclusions show up nicely. Another poster mentioned doing this on a carbon substrate. In fact, I prepared this sample twice - once on a carbon stub and once on an aluminum stub. I wanted to see how much signal was coming from below. That was mostly for EDS and did appreciably affect the images. If you had a mixture of particles only, the situation might be a little different and I would recommend the dark background of a carbon substrate. You may run up against resolution limits for BSE depending on particle size. The images may not be particularly sharp due to the sizeable interaction volume and the high currents typically required for BSE. My images were collected at 25mm WD with a sizeable current for simultaneous EDS. You could improve resolution by cutting the working distance and reducing current as much as possible while still maintaining signal strength. Warren Straszheim <wesaia@iastate.edu> 18 Apr 2007

SEM - intermittent vacuum leak

I'm completely dumbfounded by this one. I am in the process of checking for leaks on our SEM, and it pumps down perfectly one moment, then it won't even trigger PiG3. When I start to take things apart to check seals and such, then I put it back together (After finding nothing wrong) it evacuates perfectly. Then I let it sit overnight to see if there is a leak, and when I get in the next morning, it is completely at atmospheric pressure and then won't hold a vacuum and trigger PiG3 anymore. I'm a little confused at how it will hold a vacuum only the first time I pump it down after dismantling and reassembling parts of the vacuum system. I don't have a leak detector, but boy would I love one! I also don't have any gauges capable of measuring absolute vacuum vs. relative vacuum, so I have no idea what the actual measurement in torr is. Justin Kraft <kraftpiano@gmail.com> 05 May 2007

This sounds like a very difficult problem to solve, especially if you don't have a good high vacuum gauge. I would suggest you check the diffusion pump and make sure it is getting hot at the bottom and cool at the top and is staying that way. I once had an "intermittent vacuum leak" that turned out to be that I had put the wrong diffusion pump oil in the pump and it was not quite boiling. That only caused poor high vacuum, though, not degradation back to atmosphere. Also, check the quantity and quality (color) of the oil in the diffusion pump. In addition, some sensor or vacuum gauge

may be dirty or faulty and switching the vacuum off intermittently. I see no choice but to sit there until it does the bad thing while you are watching. Mary Fletcher (nee Mager) <mager@interchange.ubc.ca> 07 May 2007

Does it truly not have vacuum, or only indicate this? A possibility is that the vacuum sensors or processing circuits are giving erratic performance/faulty response and since the control of the vacuum system is based on these it can complicate the troubleshooting. Also, most scopes have a "Klixon" on the ODP column (or 2: one for overtemp and one for "hot enough to function") that are continually subjected to heat and will go flakey in time and may be shutting down the ODP. We've had a good bit of this problem over the years. Try to get a handle on the vacuum by some independent means if possible - if not a separate gauge unit, then try to read the existing raw sensor voltage where it connects to the microscope to see what is happening (this assumes you have/read schematics and can locate the places to read the sensor); or your scope may have some status LEDs on the vacuum control board that might actually be labelled? Dale Callaham <dac@research.umass.edu> 07 May 2007

I do not know which instrument that you have as you do not say? I have experienced a problem in the past where the customer behaved exactly as you have. Strip and rebuild no problem, next day no good! In this case simply turning the vacuum system off and on overcame the fault. Could this be your problem as when you strip and rebuild you turn the system off? Steve Chapman <protrain@emcourses.com> 07 May 2007

Vacuum leak update: Thanks to the many suggestions I received, the vacuum leak has been identified. I thought I'd send in a summary of the suggestions that ended up being the most helpful so those with similar problems in the future may benefit. Here is the summary: 1: Using a large quantity of various sized stoppers, isolate different parts of the vacuum system to determine where the leak may be. 2: If the system has multiple Piranni gauges (As the JSM-840 does) try switching the gauges around to make sure that it is not a bad gauge. 3: Check that the diffusion pumps are heating up. (Although this was not the issue with mine, since it was not reaching a vacuum level that would allow the diffusion pumps to begin to work, I thought I'd include it just as general reference.) Also check to make sure that the temperature sensors on the diffusion pumps are functional. 4: Short of a leak detector, spray some acetone or high purity isopropyl alcohol on the different seals. Wait a few minutes after each spray. If there is a leak in that spot, then the vacuum will increase momentarily, then go back down as the alcohol or acetone plugs the leak and then vaporizes on the inside of the system. Keep doing this moving from the outermost portions of the vacuum system to the pump connection until you find it. 5: Pump the system down as far as it will go, then seal all of the valves in the closed position. Wait. The next morning, the section with the leak will not have a vacuum in it, but the others will. Rick Becker given this handy suggestion for repairing the leak when found: If the leak is in a section that you can get around (i.e. the junction between two column sections) standard electrical tape can repair it temporarily. Make sure it is high quality electrical tape. The thicker, the better. Decent electrical tape can hold a vacuum of 10^{-9} torr. Not bad- the tape is now

holding the vacuum between the "T" fitting under the chamber (Bottom is the small vent valve, top is the chamber, and the leg on the side is a large butterfly valve into the rest of the vacuum system) and the large butterfly valve. I ordered a new gasket from Small Parts, Inc. but in the mean time, it's holding fine. As it turns out, when we re-assembled the vacuum system after carrying it piece by piece up the stairs, we must have accidentally pinched the gasket when putting the two fittings together. This caused a nick about the size of a couple of grains of sand in the gasket, which would become a problem after being reassembled when the gasket would roll slightly after tightening the screws. Justin Kraft <kraftpiano@gmail.com> 07 May 2007

STEM-EDX

I have a weird problem with my STEM-EDX results. The sample is a FIB-prepared multi-layered cross-sectional sample. The structure of the sample is around 800nm Au, 50nm Ni, 50nm Au and 50nm Ni and also 500nm AlGaN. When I put the probe (diameter about 2nm) in the Au/Ni area, there were very obvious Ga peaks. I thought it was due to X-ray scattering everywhere, but the problem is that the Ga K peak is spuriously strong in Au/Ni area compared with K peak. It seems that the Ga K intensity follows the trend of Au L intensity, but the Ga L intensity does not. When the probe was in AlGaN area, this phenomenon was not present. Could anybody kindly help me to explain why? Helen Huixin Xiu <xiuhuixin@yahoo.com.cn> 16 May 2007

The key bit of information is in the first line of your description... "a FIB-prepared..." The FIBs use a Ga ion source. You are seeing the ion implantation which always occurs (to a greater or lesser extent) in every FIB sputtered foil. I generally see ~1% Ga in most of my ex-situ liftout samples which have been milled at 30kV. Redeposition of sputtered material can contain up to 30% Ga. Henk Colijn <colijn.1@osu.edu> 16 May 2007

You should consider doing a low-energy, low-angle "cleaning" as the last step in the FIB process. This can be effective in removing much of the Ga implanted from previous FIB steps. Roger Ristau <raristau@ims.uconn.edu> 16 May 2007

EDS - carbon contamination

I am seeking input on what appears to be Carbon contamination. Here is the situation. Take a Pella 16111-9 stub out of bag and put on holder. Take another stub out of bag and sputter coat with Pd and put on holder. Do EDS on both. un-coated: wt% at% C 7.5 15 O 4.5 7 Al 88 78 coated: C 23 38 O 6.5 8 Al 71 53 SEM is Zeiss Supra 55VP with Edwards XDS10 dry scroll pump and turbo. Coater is Denton Desk IV with Edwards XDS5 and turbo. The goal of using a non-oil pumps was to reduce hydrocarbon contamination. So, where is the C coming from? Nothing has been done to the scroll pumps since they were new. There are kits for repairing them but when is this necessary and what would indicate that it be done? Would high C be an indicator? I'm stumped on this one. Any ideas? Gary Gaugler <gary@gaugler.com> 14 Jun 2007

Can't help with the uncoated stub, but most of the "C Ka" you are seeing is presumably from the Pd Mz line which is at 43.36 A (vs the nominal 44.0 A for C Ka). John Fournelle <johnf@geology.wisc.edu> 14 Jun 2007

You're pretty clever, Gary, and I'm sure you've already done this. But, have you checked for any exposed wiring, gaskets or seals near the target that might be degraded when the plasma is activated? Are you using Argon gas to vent the system and as the source of plasma? If so, how clean is the gas used to generate plasma? Sometimes, gas cylinders contain traces of oil (as might the pressure regulators). Check the threads for the presence of a lubricant. Anyone else using the system? If so, they may have left some contamination inside the chamber (like finger oils). If you are still having problems, try putting a coated TEM grid inside the chamber and examine it for traces of contamination. Sometimes, "seeing" the contamination is a clue to where it may be coming from. John Bozzola <bozzola@siu.edu> 14 Jun 2007

Thanks for the reply and to the others who have replied. More data. Gas is industrial welding Ar run through a Matheson molecular sieve (to dry and filter). SEM chamber uses industrial N2 also run through a molecular sieve. Specimens are put in SEM chamber via Fjeld M-100 specimen load lock. This unit is pumped with small oil pump and turbo. Main SEM door is rarely opened. I have to check load lock pumps to see if it really is an oil roughing pump. I thought I got a dry unit for this too. Pd target is from Refining Systems Las Vegas and is 99.5% pure. Trace elements do not include C. Coater is only used by myself. Chamber of coater is stainless steel (so it seems--but metal nevertheless). Only visible seal is the L ring rubber, or whatever, at the top. This C issue just came up while trying to quant TaN on Si. It showed C that should not be there. So now I wonder about all spectra work and quants that include C. I can't think of a way to narrow down where the C is coming from and how to negate it. I will try cleaning the stubs and also try other stub types. Exactly what are you saying about the TEM grid? The procedure is not clear to me. Are you saying I should coat it with Pd? Then what? I have STEM but not TEM. Gary Gaugler <gary@gaugler.com> <gary@gaugler.com> 14 Jun 2007

I agree with the different comments about Pd Mz line overlapping on Carbon K. I had the same stuff with new polished and ion etched Pt standards, where I found that intrusive carbon... which was Pt-N line! And my software doesn't have the N lines tabulated! I also had the problem with the Pd-M in Pd/Fe alloys. Added to the overlapping problem, in some situation one has catalysis effect on carbon cracking. The carbon peak comes really from carbon, but grows during the spectrum acquisition! In such case you should see these famous hated black squares after the analysis... About the uncoated sample, a colleague now retired said he never to used plastic bags or plastic boxes for sample storage. Most plastics outgas solvent continually and pollute all the surfaces with solvent. He used only glass ware. One needs only a monolayer of hydrocarbon to have some contamination! People who make Auger spectroscopy know that one must clean away the carbon before one see something else! I've made some test, and my conclusion is that in most cases, the sample and the sample holder bring with them the major source of contamination. The second source is the rotary-vane pump, and the last is the diff pump. The two ways to limit contamination are first to use a LN_2 cold trap very near the sample, in front of the OL, or beside, or around it (one must have a fast entry lock), and second to put all parts which comes to air, the holder and the sample in a plasma cleaner, before putting them in the SEM (one must have much money to buy one !). A simple glow discharge in air can be

NETNOTES

sufficient, but is not very reproducible. Jacques Faerber <jacques.faerber@ipcms.u-strasbg.fr> 15 Jun 2007

ELECTRON DIFFRACTION - Instability

We are beginning to explore the use of electron diffraction in the study of crystallized biological molecules. We don't have the background here. Some of these molecules are very fragile and disappear before a pattern can be captured on film. An electron diffraction pattern is seen, briefly, and then it disappears. We have been following what the microscope manual instructs on generating electron diffractions and have gotten some advice from a metallurgist. But we need some more help. Using purchased standards, we have gotten some very nice diffraction patterns. Jeannette Taylor <jvtaylo@emory.edu> 03 May 2007

You will need to work in low dose conditions. If you are using film, I highly recommend the most sensitive film available, and such films as LoDose or MRF32 X-ray films are about 20 times more sensitive than SO163. The only problem with these is that they must be handled in total darkness, so it will be difficult to cut them to size. Loading and unloading the camera, loading the racks, and developing the film are also somewhat difficult, but the skill to do that will come. Another concern is static electricity, which will make very interesting patterns on the developed film, but these will destroy the ED data. The simplest way to get good data is to insert

the selected area aperture that is the right size for the crystals you are looking at, put the scope in diffraction mode, use a very small condenser aperture, a high spot size, and underfocus the beam until you have parallel illumination, then scan the grid in diffraction mode, either looking for a good pattern or defocussing the beam and looking for the distorted image of the crystal in the zero-order spot. Blank the beam, set the lenses to the proper values to obtain a spot pattern (if you were searching in defocussed diffraction) start the exposure, and turn on the beam only when the shutter opens. This happens automatically for some microscopes, but for those that do not have a pre-specimen shutter, you must do it yourself. Bill Tivol <tivol@caltech.edu> 04 May 2007

Just a few comments off hand without ever doing these types of samples. Your problem will be similar to what happened to me with glass samples. You are putting too much energy into your sample and it is heating. There are several things you can do to help. You have to do things quickly, i.e. low dosage. You have to lower the amount of energy you are putting into the sample. The best way to do this is going to higher accelerating energy. Remember, diffraction is elastic and you don't lose energy in the sample via that route. If your energy is lower, you have more inelastic scattering and you are dumping energy into the sample with those types of scattering events. You probably are working on a 100 or 120 kV machine, but you didn't say so. And another thing that you can do is to cool your sample and use a liquid nitrogen stage. Having said this, I don't have any idea what the higher energy will do to your sample in terms of radiation damage. - Scott Walck <walck@southbaytech.com> 04 May 2007

Having done considerable ED at voltages from 100 to 1200 kV, I'd like to offer a few minor corrections to your post. You are correct that the problem is that too much energy was being deposited, but heating is not the effect that causes the pattern to decay. Changes in the specimen that destroy the crystalline order are responsible, and these are caused by breaking of chemical bonds, ionization, and other processes, such as displacement of atoms. Going to a higher energy does give advantages for ED, principally making the scattering closer to kinematic and getting higher resolution spots due to the flatter Ewald sphere. As the energy of the beam increases, the total scattering cross-section decreases, which results in less energy deposited, but the ratio of elastic scattering to inelastic scattering also decreases, so the damage-to-information ratio increases, but this effect is minor, and excellent ED patterns can be obtained at 1200 kV. Bill Tivol <tivol@caltech.edu> 04 May 2007

ELECTRON DIFFRACTION - orientation of the diffraction pattern

When you defocus a diffraction pattern, you can see a small image of the selected area in the 000 spot. When you go from underfocus to overfocus the image of the selected area is rotated over 180f. Can somebody tell me which of the two small images has the same orientation as the image in image mode? Do I have to underfocus the diffraction pattern or overfocus it? : Wouter Van Renterghem <wvrenter@sckcen.be> 04 May 2007

The orientation of the pattern is not necessarily the same as that of the image in either under or over focus, and, furthermore, it can be different for different camera lengths. There should be information in the manual of your instrument that can tell you the difference in orientations. If not, put in a specimen with an asymmetric object, take an image in normal mode, then take one in defocussed diffraction mode and compare. An aggregate of gold beads on a carbon film is a good test object for this. Bill Tivol <tivol@caltech.edu> 04 May 2007

Microscopy AND Microanalysis

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HUMOR

Dear Abbé

Dear Abbé,

I help run a multiuser facility housing several microscopes. I'm having a terrible time making users follow the rules, clean up after themselves, not break things – well, you get the picture. Do you have any advice on how to deal with these rude scientists?

Flustered in Frankfort

Dear Flustered,

Quit your whining! Obviously you have taken the liberal, passive approach to lab etiquette and training. You need the "Lab Bat" by RonCo. I found that a few judicious whacks with the Lab Bat will straighten up an uncooperative user quicker than you can say "Bremsstrahlung". It's also great for attitude adjustments, encouraging productivity, and improving morale. Of course there is some advice I would offer for Lab Bat usage. Don't whack the hands – those are important for continued productivity and leave visible marks. Of course I have popped someone upside the noggin when it's obvious that cranial injury will have little effect on the mental acuity of the user. While I prefer the solid thwack of an oaken Ladd Lab Bat, some enjoy the metallic twang of the Knoll Titanium models. It's all a matter of personal preference.

Dear Abbé,

I am in charge of purchasing a suite of light microscopes for a new multiuser facility. Unfortunately the budget provided by the college is not as large as I had hoped for and we can only afford a few quality microscopes with limited capabilities. Do you have any suggestions for microscopes with large assets and less cost?

Cheapskate in Chicago

Dear Cheapskate,

My many years of experience tell me that with microscopes, as with most things in life, you get what you pay for. Sometimes you get less. One time in Bangkok I got a whole lot less, but I digress. If all you want out of life is something with the maximum in magnification and big focus knobs to twiddle then fine, go ahead, and waste your money on a room full of shiny trinkets that can be strutted out in front of the Board of Trustees like so many cheap Stabmädchen smelling of cheap perfume and cigarettes. But if you want a REAL microscopy experience then you should focus on obtaining the greatest resolution no matter what the cost. Better to have twenty or thirty students standing patiently in line awaiting their turn at a real instrument than to give everyone their own inferior equipment. Such cheap instruments can only lead to disappointment, heartache, and the occasional communicable disease.

Don't hold back! The old, tired saying goes that "there are no stupid questions." But if you need to find out, please contact Herr Abbé's administrative assistant at jshields@cb.uga.edu.