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NetNotes

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Specimen Preparation:
osmium

Could others please share their experiences with the premade aqueous OsO_4 solutions? I have been making my own 4% solution, storing it in the fridge, and diluting a small volume just before use. However our EM lab isn't very busy (3-4 preps every few months, although I am working on trying to get more users!) and I end up having to throw out a lot of the 20 ml solution that I make; membranes start looking kind of crappy and with the older solution even if the solution still looks relatively clear. I also see a lot of black specks in my unstained sections. I don't know of anyone who uses the premade ampoules, so I don't know if there are any significant differences in image quality. They come in such small volumes, which would be really convenient for me! ***Blanca Carbajal Gonzalez bicarbaj@mtholyoke.edu Fri Sep 18***

For the reasons you mention, we have been making use of the premade aqueous OsO_4 solutions for years. It works well for our own use and it is convenient when another user comes by asking for a few milliliters of OsO_4. Occasionally we still make up a solution for odd ball recipes or large quantities. Louie Kerr lkerr@mbl.edu Fri Sep 18

I worked in biological microscopy for several years prior to a long career in an industrial polymer microscopy. In the bio lab, we invariably made our OsO_4 solutions from crystalline OsO_4. In the polymer microscopy lab, we rarely used OsO_4, often in short bursts. Invariably, I was skeptical of the pre-made solutions because many of the vials went to waste as indicated by black precipitate on the inner surface of the vials and loss of amber color of the solution. The issue, as you indicated, is quality versus price (through waste). To maintain high quality, I suggest you keep OsO_4 crystals on hand and make fresh batches as needed. The price of the crystals is less than the prepared solution and you can be assured of the quality: Electron Microscopy Sciences sells 1 gram of OsO_4 crystals for $32 versus $53 for 10, 2 ml vials of 4% OsO_4 solution. When you make up a batch OsO_4 solution, break it up into several small vials and store each under an inert gas (nitrogen or argon) head to slow degradation of the oxide. When a vial begins to go bad, hopefully the unopened vials are still fresh and usable. Gary Brown microscopy.gmb@gmail.com Fri Sep 18

As a matter of curiosity, what is the consensus on the usable lifetime of a made up from crystal, 4% osmium in water stock solution, stored in a fridge? While on the subject of osmium stock solution storage, do people clean their stock solution storage bottle between making up their osmium stock solutions? Allan Mitchell allan.mitchell@stonebow.otago.ac.nz Tue Sep 22

I've not seen yet (I don't think?) any mention of freezing the osmium samples. Is there a reason for this? In this facility, I make up fifty 1 ml aliquots of 2% aqueous osmium, from 1 g osmium crystals, into clean 7 ml flat bottom glass vials with polypropylene screw cap lids with foil inserts (to prevent splashes and vapor seepage), and store them in the -20°C freezer in double Tupperware boxes (one inside another - for extra safety) only taking out the required number of vials for each processing session. As they are such small quantities, they are safer to handle and defrost quickly. I have found that I can store the osmium in this way for years without noticing significant artefact. I would also be interested to hear how people neutralize their osmium after use. Nat Allcock nsa2@leicester.ac.uk Wed Sep 23

I always reduced osmium tetroxide and ruthenium tetroxide to their dioxides using a sodium bisulfite solution (10 wt/vol %). Add an excess of the bisulfite solution into the vial of the tetroxide and leave for a while, an hour or more. This works well. Some labs reduced osmium tetroxide with unsaturated vegetable oil. Gary M Brown microscopy.gmb@gmail.com Wed Sep 23

Specimen Preparation:
carbon coating

What would anyone out there recommend for a TEM grid carbon coater for ultraclean carbon deposition (dry/UHV system), with precision in the 1-5 nm thickness range and a real-time thickness monitor? ***Larry Scipioni les@zsgenetics.com Thu Sep 17***

For carbon coating, you may prefer to use a turbo-pumped system and a fast evaporator. You may have a try on two systems which I have experience with: - Cressington turbo coater 208 (carbon rod and e-beam guns are available) - Leica ACE series (carbon rod and carbon wire are available, to my knowledge; e-beam?) other machines from other companies will do the job as well, I assume. Quartz thickness monitor yes; but real-time thickness: you will have to wait for a few (5 to 10) seconds until the system settles (physics of heat transfer). Upon carbon evaporation, the quartz is getting heated as well and will give you some numbers which are not realistic. At the end, we find this reproducible. Both machines work fine, in our hands, for light shadowing of bio-samples for STEM, TEM or SEM. try to find a lab where you can have a test experiment with your samples, or convince the sales rep to give you a system for a week for several tries. As you do not explicitly state what you are going to do: "TEM grid carbon coater": if you want to produce your own carbon supporting film, you may have first to shadow carbon onto mica, then float this off (e.g., on a water surface), and then pick up the C-film with (hydrophilized, glow-discharged) grids. Reinhard Rachel reinhard.rachel@biologie.uni-regensburg.de Fri Sep 18

Specimen Preparation:
bakeout chamber for cleaning STEM samples

I've been trying to develop a procedure to clean our STEM samples prior to insertion into our ARM200CF microscope. In particular, we are trying to perform highly analytical work that involves long EELS / EDS mapping, so we would like to reduce hydrocarbon contamination on the surface of our samples. Since most of our specimens are oxide lift outs, they are quite robust to heating and plasma cleaning. Typically

after a lift out is completed I will plasma clean for 2-5 minutes prior to insertion; however, someone recently suggested baking our samples for a few hours at 50-100°C to further reduce contamination. It was also suggested that we place our heater in a bell jar-like setup so that we can pump on the sample during the annealing. I am considering building such a setup, but I would like to see if anyone has constructed something like this before. I would very much appreciate any design suggestions or other tips to help us clean up our samples. ***Steven R. Spurgeon steven.spurgeon@pnnl.gov Tue Sep 2***

It definitely helps to bake—and not be shy about it. 160°C for 8-12 hours (i.e. overnight) is good. I find that if that doesn't do it, it won't come clean. Our setup is not exactly home-made, but it consists of a Pfeiffer HiCube dry pumping station and an MTI quartz tube furnace. That way it is possible to do vacuum or air or to introduce other gas environments. And the temperature vs. time profile is programmable. I can send you a picture if you're interested. May be overkill for you. Larry Scipioni les@zsgenetics.com Tue Sep 22

Have done this using a turbo vacuum system we used for pumping holders, with a tube connected, covered by a vacuum flange with an electrical feedthrough (used parts of an old Penning gauge for that). Inside I clamped a 12 volt DC halogen lamp to produce heat. Our mechanical workshop machined a small open aluminum grid box which holds 16 grids. This goes into the tube, pump, ramp up the lamp, wait some hours, vent with clean nitrogen. The small dry turbo system is going to be main cost. The other parts can be cheap. You might also consider putting a UV source in. Wim Hagen wim.hagen@me.com Wed Sep 23

I wanted to build a similar system for drying of NMP from my TEM grids. I just hooked up a Peltier cooler/heater with an old desktop PC power supply and controlled it with a mechanical relay, which in turn was controlled by Arduino Uno. Temperature feedback was provided by a LM-35 sensor, which I got in an Arduino starter kit. Peltier heater and sensor was kept in a vacuum oven which had few holes drilled to make way for wires which were then sealed with epoxy resin. It was the cheapest and quickest way to do it and it can easily maintain a temperature of 60°C ± 2°. For higher temperatures and better control, you might try better heating coils, thermocouples and a proportional-integral-derivative (PID) algorithm. The rest of setup should remain the same. Amit Gupta amit.welcomes.u@gmail.com Wed Sep 23

Specimen Preparation:
holey carbon support film

Has anybody prepared their own holey carbon film supports? We usually use the film on Cu mesh grids. How is the mesh grid manufactured? What if I need it on a polymer grid? How do you control the size of the holes? What is the range of the holes we can achieve? ***Zhaoxia Zhou z.zhou@lboro.ac.uk Wed Sep 23***

I have done so, and it is trickier than making films without holes. There are several protocols that work, but it may take some time to develop the necessary skills. I found the trickiest part to be the separation of the holey Formvar from the glass slide. Commonly, the oil from the skin of one's nose is used to coat the slide before dipping in Formvar; however, in my case this oil did not work—it did work for former without holes. A solution of Apiazon L in petroleum ether did work for holey Formvar. Another difficulty was that separating the film from the slide did not work when the humidity in the lab was high. Bill Tivol wtivol@sbcglobal.net Wed Sep 23

Years ago, back when dinosaurs still roamed the earth, I made the holey Formvar films for our lab. We didn't use carbon films back then in the biological microscopy lab. I used the method described by M. A. Hayat, *Principles and Techniques of Electron Microscopy (Biological Applications (Volume 1)*, Van Nostrand Reinhold, 1970, 324–332. The method for production of Parlodian (nitrocellulose) or Formvar (polyvinyl formal) plastic films is straightforward but requires practice to learn the technique. The method is the same for production of continuous and holey Parlodian and Formvar films except that glycerol is added to the Parlodian or Formvar solution prior to casting the films; the glycerol is insoluble in the solvents used to dissolve the plastic films. The size of holes in in holey films is proportional to the concentration of glycerol in the mixture: 12% (vol/vol) glycerol gives holes with a maximum diameter of approximately 25 µm whereas 0.08% glycerol gives a maximum hole size of 4 µm. Although you can learn the method of holey carbon film preparation, if you don't do it often, the process becomes very laborious and time-consuming. I suggest you consider purchasing your holey carbon film grids from an electron microscopy supply house. That's what I did for the last 25 years. The Hayat book can be found for $8 - $35 on Amazon. Regarding your question about TEM grid manufacture: metallic TEM grids are electroplated. I don't know how non-metallic grids such as nylon are prepared. Gary M Brown microscopy.gmb@gmail.com Wed Sep 23

Specimen Preparation:
Lowicryl K4M or HM20

I need to prepare some Lowicryl. I am leaning towards K4M. I have never done this. Can someone give me a few pointers? The recipe says a brown glass container. But these are pricey containers to purchase for mixing up resin. I would like to use this in my freeze substitution device on some heart tissue for immunolabeling. If I am using ethanol in my resin mixtures, should I use ethanol in my substitution medium instead of acetone? ***Chris Brantner chrisbrantner@email.gwu.edu Wed Sep 30***

I have used Lowicryl K4M, LR White and Gold and HM20). I have found HM20 to be superior in: infiltration, preparation, sectioning and immuno-labelling. I would recommend it over the others, you can make it up in a glass scintillation vial (mixing with light N_2 bubbling). I have also found that acetone is the best solvent for IEM, I have tried both methanol and ethanol-too much extraction. We have done the HPF hybrid technique (light pre-fixation in phosphate buffered paraformaldehyde-rinse-HPF in hexadecene-LN_2 storage), followed by AFS. Quickly my protocol is AFS in IEM cocktail (0.2% GA, 0.2% UA in 95% Acetone (5% D-H20) at -90C. This is followed by gradual increase (slope 5 degrees/hr) to -45C, then held there for solvent rinse (3×10 min pure Acetone) followed by 50% then 75% HM20 in Acetone. After an overnight in pure HM20, I change to fresh resin once (2 hrs) then again right before UV illumination. Sections are picked up on Formvar-coated nickel or slot grids and immuno-labeled by floating on drops of: NH_4Cl, block, primary antibody overnight 4°C, TBS rinse, gold-conjugated secondary antibody (2 hrs RT), rinse, hard glutaraldehyde fix, rinse, uranyl acetate stain rinse and dry. No first Aby sections followed by secondary Aby serves as negative control. The key is to make sure the tissue blocks are small and fit into the planchets, we usually use two 300 µm.depth planchets filled with 1.2 µl of hexadecene. When sectioning, due to the fact that a good freeze is within a 200 µm depth of the tissue, we concentrate on the periphery of the tissue, not the middle. Michael Delannoy mdelann1@jhmi.edu Thu Oct 1

Specimen Preparation:
Unicryl sectioning

We are trying to do post-embed immunolabeling of cell pellets in Unicryl for a client. However, we are having difficulties sectioning her blocks. The blocks are extremely hydrophilic; even if the boat is very under-filled, water is still attracted to the block face and pulled over the knife. Any sections that we get are pretty much unusable. I have experience sectioning K4M, LR White, and Unicryl before, and the hydrophilicity has never been this bad. The protocol we used is as follows: Fixation in 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer. Rinse in phosphate buffer (3×15 minutes). Dehydration series of 25%, 50%, 75%, 95%, and 3 100% changes with a freshly opened bottle of ethanol. Infiltration of 1:3, 1:1, 3:1 of 100% ethanol and Unicryl. Ethanol was same freshly opened bottle. 4 changes of 100% Unicryl (2nd one was overnight). Polymerization in Eppendorf tubes in a 55°C oven for 1 day, then a second day once we started having sectioning issues. Some troubleshooting we have done at the microtome includes: Varying the cutting speed and microtome arm cycle speed, varying the thickness setting of the microtome arm advancement, using a new knife and new section of the knife edge, varying the water level from slightly under-filled to severely under-filled, and varying the block face size and shape. Any advice would be greatly appreciated. ***Erin Stempinski erin.stempinski@nih.gov Thu Oct 8***

I have also used K4M and HM20, one being hydrophilic and later hydrophobic. I have switched to HM20 for the same problems you cite. I would eventually get sections with K4M, but not without a lot of duress. I wonder why you polymerized in an Eppendorf and not BEEM capsules. The container must be air tight and I know some Eppendorf's can leak. Also make sure you are adding enough catalyst, by weight not volume as viscous liquids will stick to any pipettes etc. Also I believe the K4M can be polymerized with up to 5% by weight water, so I am curious as to why you are going through 100% ethanol? I can typically stop at 90% ethanol and mix (i.e., LR White) as my first infiltration step, especially since your protocol does not protect membranes (you can use tannic acid and *en bloc* uranyl acetate staining for that). If the second day of curing helped your problem, then it's a polymerization issue. Michael Delannoy mdelann1@jhmi.edu Thu Oct 8

Here's some additional information: Dehydration steps were 15 minutes with 95% ethanol overnight. We dehydrated to 100% for two reasons: 1. We have used this protocol with success previously for this target and 2. Ultrastructural preservation/sectioning was really poor in past experience when doing post-embed immunolabeling with plants and LR White if the sample was not completely dehydrated. We used the Eppendorf tubes because the pellets were very small and delicate (one was just a small smear of cells along the side of the tube) and we were worried about losing them in the transfer to gelatin capsules. The sheet on Unicryl on EMS seems to indicate that they should polymerize in the Eppendorf tubes just fine. Is that not what other people are experiencing? We are currently polymerizing even more by UV at 4°C. We also got a suggestion to place the samples overnight in Dri-rite which we will also try. Erin Stempinski erin.stempinski@nih.gov Thu Oct 8

Specimen Preparation:
TEM and SEM on same sample

Has anybody done TEM after SEM on the same sample (talking about soft animal tissue) with good or acceptable morphology? ***Yorgos Nikas eikonika@otenet.gr Wed Sep 16***

It will depend on tissue and parameters of treatment prior to as well as fixation, processing for SEM, observation in SEM and then parameters of reprocessing specs for TEM. It can be done and can be a valuable supplementing info to the images documented by SEM. (will send you an old EMSA-MSA-abstract [1990, with images] with an example of "arterial" SEM to TEM by separate personal mail), Wolfgang Muss w.muss@salk.at Wed Sep 16

Yes. EDS, even. I just had the sections (thin sections) mounted on the TEM grid ready for the TEM, then put them in the SEM. This works best if the SEM stub has hole drilled in it just less than 3 mm diameter, so that there is a void space below the grid with samples. Be careful though. The lower kV used in SEM will result in greater beam-stopping by the sample, therefore more energy deposited in the sample and a greater likelihood of rupturing the section or any supporting film. Phil Oshel oshel1pe@cmich.edu Wed Sep 16

I should add that I've also done SEM-then-TEM on tissue samples prepared for TEM, *en bloc* stained or not, then dried and sputter coated for SEM. After the SEM, "rehydrate" in 100% ethanol, embed and section for TEM. The morphology in the thin sections likely won't be as good as tissue processed for TEM, embedded, sectioned, stained (or not), but depending on the study, it can still be useful. Imaging something like (vertebrate liver) bile canaliculi first in the SEM, then in sections in the TEM is a good example of this. Note: the sputter-coated layer of metal causes no issues when sectioning. Phil Oshel oshel1pe@cmich.edu Wed Sep 16

I did a lot of this in the 1980's on human ovary. We were looking for particular epithelia cell populations so we would find the cells in the SEM, dissect out the area and then do TEM on that area. As a control we also worked backwards. If we saw the cell population of interest in TEM sections, or semi-thin sections, we would then dissolve out the embedding resin and look at the block in the SEM. Worked quite well and the work was published. It was related to damage done to the epithelia layer of the ovary during surgical procedures. The ultrastructure in the TEM after SEM preparation wasn't super good but it gave the information that was required. This was all pre-PDF and pre-computers so give me a few days and I will dig out my old notes, scan them and send them to you. Allan Mitchell allan.mitchell@stonebow.otago.ac.nz Wed Sep 16

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LS7 Organism development and imaging
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INSTRUMENTATION AND METHODS (IM)

IM1 Tomography and Multidimensional microscopy
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MATERIALS SCIENCES

MS0 Nanoparticles: from synthesis to applications Interdisciplinary session, common between life and materials science
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Specimen Preparation:
chitin

Can someone lead me to a good set of protocols for fixing chitin? My biology friends tell me that conventional methods for fixing chitin often fail to adhere the plastic to the fibers. I am doing an analytical study of the inorganic components in the chitin so I don't want to use any staining and I want to preserve the integrity of the Ca phases present. Thanks from a materials oriented microscopist! ***Ken Livi klivi@jhu.edu Wed Sep 16***

I work on cell walls of plants not fungi. But I believe the issue is related. From my reading of the literature (and this is far from complete), classical chemical crosslinking fixatives, like formaldehyde and glutaraldehyde, react to only a limited extent with cell wall polymers. I have always used fixatives when I want to keep the tissue or cytoplasm in good shape. But the wall itself I think does not need fixation (except perhaps to inactivate cell wall degrading enzymes that live out there). Cell walls are tough and can withstand typical dehydration / embedding schedules without too much trouble. You wrote about plastic adhering to the chitin. This seems like more of an infiltrating/embedding issue. An old trick that works to embed certain tricky plant samples is to start infiltrating with really low concentrations of your plastic, like 1% then 2%, 5%, 10% and then as normal. Tobias Baskin baskin@bio.umass.edu Wed Sep 16

Any chitin in particular? Arthropod cuticle, fungus, or somebody else? This is a ubiquitous polysaccharide, but not necessarily identical. Especially for minerals, like Ca. And: Your question implies you're wanting to do TEM, but perhaps SEM would be better? If you're not interested in the ultrastructure of biological components (like cells), you could let the critter dry, then cryofracture in liquid nitrogen. This would expose the internal structure of the chitin and contained mineralized phases. If you need a polished surface for x-ray spectroscopy or EBSD, then this wouldn't work. Phil Oshel oshel1pe@cmich.edu Wed Sep 16

I think that (at least some kinds of) chitin have a waxy coating. If that is true of your chitin, Ken, and if the wax structure is part of what you're interested in—it may contain some Ca—any additive that could dissolve the wax will be problematic. Perhaps Phil's suggestion of SEM to characterize the wax (if any), then prep for TEM would be best. Can chitin be placed in the SEM without treatment and/or coating? If so, then I'd definitely try it. Bill Tivol wtivol@sbcglobal.net Wed Sep 16

Waxy epicuticles are common in insects and terrestrial arthropods, but that's about it. Not part of the chitin per se. And any wax has an unfortunate tendency to melt under the beam. Cryomethods are needed to study it - and the wax on plant leaves. That said, waxy coats on critters and plants are very poorly studied and I'm sure have more uses than just slowing or stopping dessication. Phil Oshel oshel1pe@cmich.edu Thu Sep 17

Specimen Preparation:
SEM of leather

I'm looking for suggestions on how to prepare leather for SEM viewing; tanned & untanned, tanned is worse for charging. Currently I slice a cross section by hand as thin as possible. Mount with a small dab of Duco cement, and silver paint the edges. (Tried mounting with a dab of silver paint- not much improvement). The corium takes gold coating reasonably well but the grain seems to be resistant to all but long sputter coating. I get charging at higher magnifications. Would a different metal coating do better? Perfuse some conductive material into the sample? Although that might disturb structure. ***Joe Uknalis joseph.uknalis@ars.usda.gov Mon Oct 5***

My first question would be what accelerating voltage are you using, and what spot size when compared with your normal operating conditions? With this information we may move forward. Steve Chapman protrain@emcourses.com Tue Oct 6

The Duco cement isn't helping you—you're mounting with an insulator. I would use a conductive carbon tab/tape and silver paint around the edges. Then sputter coat, using 60/40 gold/palladium. Cheaper than pure gold and it gives a better (finer grain coat). More importantly, what kV and spot size are you using? Reducing both will go a long way to reducing or eliminating charging. This may be all you need to do. You could also try adding osmium vapor "fixation". Place the sample in a petri dish and a crystal of OsO_4, or a drop of 2-4% OsO_4 in water in a smaller dish, so the OsO_4 doesn't contact the sample. Don't cover the same dish with the OsO_4 but cover and Parafilm sealed the big dish. Let sit at room temperature overnight to 24 hrs. The OsO_4 doesn't bind that well to proteins, but it will still bind. Phil Oshel oshel1pe@cmich.edu Tue Oct 6

I was using 10 kV spot 3 on a FEI 200 FEG I dropped to 5 kV but the charging got worse? Unfortunately I don't have a carbon coating option. Low vacuum/ESEM has poor resolution at 50,000×. Will try the osmium vapor. Gold/palladium is more $$ than gold alone, may give that a try. Will try different spot sizes. Joe Uknalis joseph.uknalis@ars.usda.gov Tue Oct 6

I would start at about 800 V and work my way up. I try to get to the point where the electrons in (beam) are close to the electrons out (SEI, BSE, etc.), and I have the best resolution I can, with minimal charging. With the FEG, I think you will be able to get the mag you want, though I am not familiar with your SEM. I start at low kV and work my way up. If you start high and go down, you will never see that area as best as you could have. Darrell Miles milesd@us.ibm.com Tue Oct 6

Sounds as if you are trying, but if the charge is worse with dropping to 5 kV that tells me you have lost the BSE contribution, this will be a big problem when using a through the lens (TTL) system. The converted BSE provide SE, but because they are derived from BSE they do not carry so much charge information The approach to a problem specimen should be as follows - 1. Use the smallest possible piece of material, fixed to the stub with a good quality conducting media. 2. Start at the lowest kV that you are able to work with, and a small spot size; all aimed at not damaging or charging the specimen. If you use a TTL system go to the standard detector in the lower chamber, with about 7 mm working distance. You need a contribution of converted BSE (less charge) which you are unlikely to have with TTL detection. If the signal is too low, slightly increase the spot size. 3. If the specimen is not giving you a problem other than resolution, increase the magnification using a fast scan if possible, to reduce the intensity of beam on a particular area. 4. Having increased the magnification, as resolution is the problem, increase the kV slightly (e.g. if you were using 1 kV go to 1.5 kV). 5. As long as there is no charge continue stepping up the kV until you are happy with the result or the specimen charges. If you charge the specimen take it to air to discharge it, and go back to an earlier kV. Slight charge may be reduced by reducing the spot size slightly. 6. If resolution is still the problem, try moving the specimen closer to the final lens by a few mm, not too close as you will lose the very valuable converted BSE. 7. Still a resolution problem, if you have a TTL system try 10 mm working distance (WD) then in steps down to 5 mm WD with this system, and see if you are able to obtain a satisfactory result (with many TTL systems between 7mm and 5mm works). 8. All of the time you are looking for a mix of kV, spot size, and WD that provides the best mix of signal and therefore performance. Steve Chapman protrain@emcourses.com Wed Oct 7

I agree with what Steve had to say and have some additional comments. A sample of a square centimeter or so is probably not too large. The conductive media should make a continuous electrical connection between the bare metal stub and the upper surface and continuing onto the upper surface of the sample. I often use Leit-C carbon adhesive from my friendly microscopy supply house because it combines electrical conductivity with excellent mechanical strength that materials like colloidal graphite (DAG) and silver paint don't have. Silver paint can also be problematic in EDS analysis. — The power that field emission SEM offers is incredibly bright source combined with a stable low voltage beam. Secondary electron imaging (SEI) at low voltage provides the best topographical analysis of surface roughness. Charge control in SEI is achieved by balancing the number of incident electrons entering the sample and electrons leaving as secondary and backscattered electrons. I highly recommend the book "Scanning Electron Microscopy and X-ray Microanalysis", 3rd Edition by Goldstein et al., Springer, 2013. The chapter on Procedures for Elimination of Charging in Nonconducting Specimens should be very helpful. — My 25 years of experience in low voltage, field emission SEM of polymers leads me to believe that some of the imaging problems experienced with leather may be similar to those observed in polymers. Both are insulators and consist of low atomic weight elements. Like Steve, I suggest you start at around 1 kV accelerating voltage. Use the spot size that the service engineers use to check your spatial resolution at 1 kV: this spot size is a good place to start because the manufacturer knows it gives an excellence balance of resolution and signal-to-noise. For best results, I suggest you work with an uncoated sample. You might start with a coated sample then move to the bare, uncoated sample. Gold and gold-palladium coatings will obscure possibly important surface information. Faster scan rates produce less charging than longer scan rates. If a single scan allows you to control charging but is somewhat noisy, average several scans recorded using shorter scan rates. Do a few quick tests on fresh areas of the sample to see how long a scan rate you can use without producing charging. Be aware that your default image acquisition scan rate may be much longer than the ideal scan rate for your sample. If this happens, record your images at the preferred rate. Fortunately, sub-micrometer surface texture and porosity (as I would expect to see in collage-based leather surfaces) dissipate charging thus making imaging easier. Gary Brown microscopy.gmb@gmail.com Wed Oct 7

LM:

demonstrating the field and aperture

For those of you teaching microscopy courses, it can be challenging to explain the field and aperture diaphragm and how they affect the cone of light emerging from the condenser. On a whim, I filled a cuvette with fluorescein in order to visualize the cone of light. The demonstration proved quite successful, so I made a movie of the demonstration, showing how the field diaphragm affects the width of the cone without impacting its angle, and the aperture diaphragm impacts the angle of the cone without affecting its width. Having the students see the cone also went a long way in helping them understand why axial resolution goes down with a lower NA. Here is the link to the movie: https://youtu.be/06CQ6IIaDWs The cuvette also allowed me to show the hollow cone generated in phase contrast and darkfield: http://imgur.com/hswMRjH http://imgur.com/Zl87dcr The sectored ray in oblique illumination: http://imgur.com/oerMkk0 As well as the solid cone in brightfield: http://imgur.com/KZP3Sv0 http://imgur.com/akeiI0T Small disclaimer, the oblique was a "poor man's" oblique done my misaligning the darkfield annulus and partially closing the aperture diaphragm, but I also like to show "poor man's" oblique to students to show how thinking a little outside the box can allow you to get the most out of your microscope. ***Ben Smith benjamin.smith@ou.edu Wed Sep 23***

Well done, Ben. These are such valuable lessons to learn. Your movie was especially effective. When we are on the road, we often use a business card for this demonstration, placed perpendicular to the stage. Also, I have a piece of screwdriver handle stock (about 1" in diameter), polished on one end, and placed on a slide with a drop of oil under it. In the old days, we used to use uranium glass. I understand that Jerry Sedgewick (jerry@imagingandanalysis.com) has a new supplier, so that approach may also come back into vogue. As for "poor man's oblique": unless one has a condenser with an Abbe slider, I doubt that any of us could have done better. No apologies necessary! So here's question to put to your students: What impact does off-setting the zero order have on resolution? Barbara Foster bfoster@the-mip.com Thu Sep 24

EM:

converting diffusion pumps to Fomblin

Could someone please share, or point me to good resource on choosing fluids for diffusion pumps? Maybe even cross-reference table for Fomblin and hydrocarbon oils? I am trying to return an old Electroscan SEM back to life and considering charging Fomblin instead of mineral oil into its Varian M-2 diffusion pumps. ***Valery Ray vray@partbeam-systech.com Tue Sep 15***

Chapter 5 of my book "Vacuum Methods in Electron Microscopy" (Wilbur C. Bigelow. Portland Press, 1994) contains a rather extensive discussion of the characteristics of various common diffusion pump fluids, plus a discussion of the factors to consider in choosing a fluid. Wil Bigelow bigelow@umich.edu Tue Sep 15

Fomblin is great if you are pumping something reactive like oxygen or fluorine. But for an EM you just want something that is low backstreaming. Look at something like Santovac 5, that is what is used in JEOL scopes. It is pretty spendy. Also look at adding an alumina-media fore line trap, this will help stop mechanical pump oil from back streaming into the scope during roughing and contaminating the Santovac. I did the expensive route and replaced the diffusion pump with a Varian turbo backed with an Edwards oil-less scroll pump. I think those two pumps are worth more than my SEM. Jerry Biehler jerry.biehler@gmail.com Tue Sep 15

TEM:

fluctuating room temperature

We noticed changes of temperature in the TEM room, so I installed a thermometer. The temperature fluctuates by approx. 3°C (21.4-24.1°C) in the day or overnight. We have a Tecnai G20 and a powerful air conditioning system. I know that temperature should be as constant as possible but I don't know how much it is important. What inconvenience should I expect with such a temperature fluctuation? What are the specifications of this instrument? ***Stephane Nizets nizets2@yahoo.com Thu Sep 3***

The specifications of your microscope allow a maximum temperature change of 1°C/24 h, 0.5°C/h, or 0.1°C/min. One problem you might experience because of "very" fast temperature changes is specimen drift. Guenter Resch lists@nexperion.net Thu Sep 3

Sure the room temperature may have an effect on TEM performance over time, but that mainly relates to the power supplies and electronics. Provided your room temperature falls within the manufacturer's specifications you should not have a problem. I have worked with instruments in hot countries well outside the recommendations and still not had problems. If we consider the average exposure time, then temperature stability over that time is not a problem.

There are many parts of the instrument that shield you from room temperature interference; general lens bulk, water cooling etc. You are more likely to suffer from magnetic field problems than any other in the modern day environment. My only advice, if you worry about specimen stage stability, is to store the specimen rod in the microscope! In this way the few seconds it will take to change the specimen will hardly change the rod temperature, and by the time you are settled in to record images, the rod will be back at "stage temperature". I have always thought it to be an operating error to store the specimen rod at room temperature, when it needs to be at specimen stage temperature. Working at very high resolution the most unstable unit in my experience is the specimen rod. Remember it is a directional object so a constant drift direction will be produced. In the good old days, when we used a round specimen cartridge, the specimen drift problem was very rare, due to the heat transfer being in all directions rather than one! Steve Chapman protrain@emcourses.com Thu Sep 3

The effect on your system will depend on what you are trying to do with it. If you are using the instrument at moderate magnifications and not using STEM or EELS then you won't see too many problems. The change in temperature relative to your column and electronics temperature will induce alignment as well as specimen drift, for STEM, HRTEM, EELS, etc. this will cause you to lose performance and have issues with stability over extended periods. You say you have a "powerful" a/c system. The issue may be it is too powerful. A/C systems are designed to generate a stable temperature by operating continually with a certain load; if the load it too small it will constantly cycle on/off cooling causing the exact problem you have. You can test this by increasing the heat load in the room (say a few bar heaters) and seeing if you get an improvement. Not a very green long term solution but could help you justify a change. Another option is putting computer servers in the room as well, these generate lots of extra load. Another potential problem is the location of the temperature sensor relative to the room airflow, if it is directly in line with the output flow it could be over cooled, tricking the system into thinking it needs to shut off and on. A "ductsock" on the a/c out will take away any heterogeneous flow and minimize this problem. Matthew Weyland matthew.weyland@monash.edu Thu Sep 3

TEM:
water condensation problem

I'm seeking advice about water condensate inside the console (column and electronic electronics). It seems that there was a brief power interruption during my 10-day vacation, and there was no alternative person to watch and report this incident. When I returned and entered the lab, I found water all around the flooring and console. The power supply was on, the water chiller was operating, the scope was inactive, and the PC had rebooted. On seeking advice from the support center, I was asked to shut the chiller and cut the power to the machine. The approach seemed to be to dry out all of the system for two or three days and then attempt to diagnose the resulting damage. ***Mohammed Yousuf mdyousuf@qu.edu.qa Mon Sep 7***

I do not know if this will help but this is my experience. Twice in my career I have had water pour all over an instrument; one from a bust tap creating a fountain in the microscope room, the other from a fire on upper floors, and the extinguisher water ending up on the microscope. I found that with clean water going all over the microscope, after a week of drying out, we had no problems. With the fire water damage, waiting the same amount of time one circuit board suffered, this was due to the contamination of the board by media from the building itself being brought down onto the board surface. No matter how much cleaning I tried I could not persuade the board to run. It had to be replaced. So clean water should not be a problem, dirty water probably is! Steve Chapman protrain@emcourses.com Mon Sep 7

TEM:
sampling

An ongoing, hair-pulling dispute between me, the microscopist, and almost every person doing research: "Why did you take so many photomicrographs for each sample? Wouldn't you say two or three photographs would be just as good as the 5 to 10 you took? I, the investigator, still can't get over you embedding 6 blocks and sectioning all those blocks as semi-things!" How do you handle this scenario? How do you demonstrate that more in EM is actually better? ***Connie Cummings ultrapathimaging@gmail.com Tue Sep 15***

I draw them pictures (I always draw pictures, as if for a child) of their sample size. For TEM, I draw a cell. Then I draw the nucleus and maybe a few other organelles. Then I draw the object of interest, particularly if they are looking for a virus. Then I draw a line through the cell, representing a 60-80 nm section, which clearly misses any of the object of interest. Then I draw a bunch more parallel lines, representing lots more sections, still missing the object of interest. If it's a cell pellet, I draw more cells, but show that, unless there are a lot of cells and they are ALL showing the object of interest, my paltry 1 mm×2 mm×60 nm section is going to miss it. If it's a tissue, same problem, probably worse. Explain sample size. For semi-thins, same problem but at a slightly different scale. I just serial-sectioned a small marine organism for somebody who had a pre-conceived notion of how something worked based on a couple of random sections taken from a paraffin block ages ago. And gave a talk about it. The serial resin sections clearly showed something else was going on altogether, and then the intermittent ultrathins blew it all out of the water. This is why I really encourage investigators to come and do their own microscopy, so they have a better idea of the context. If they don't, I take a zillion micrographs and send them all. I hate just giving them a few select micrographs that may or may not reflect my bias (me, biased?). Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Tue Sep 15

If you are digital, the memory for the images is practically free! Any professional photographer these days just shoots gazzilions of pictures. Been to a wedding lately? The photog just sits there, clicking away. My last vacation, I took 1,354 photos in 2 weeks. After I got home, I realized it was not enough. Experiments are expensive. Specimen prep is expensive. Once you have the object to photograph, take as many photos as you can! John Mardinly john.mardinly@asu.edu Tue Sep 15

One word: statistics. If that doesn't work, ask them what a sample is. The cell within which the Thing is hidden? So how many cells need to be sampled? From how many different tissues/organisms because the cells vary among tissues and organisms. The Thing, because they need to know the Thing's ultrastructure, which may vary by the local micro-environment? How many Things from how many different micro-environments need to be sampled? Perhaps it's the tissue, so how many bits of tissue need to be sampled? Phil Oshel oshel1pe@cmich.edu Tue Sep 15

Your conversation is so refreshing! Connie loves her preparations and shoots many photos, Tina points at the diverse aspects of each sample and John mentions how easier is to get photos in our digital era. So where is the problem if flooded with photos? Are they not all of them unique snapshots of each sample? Why

is a researcher bothered by them? Well, the answer is simple but sometimes difficult to say. To appreciate morphology needs affection, brain and time. That makes it less attractive to people inclined to approaches that are easier to interpret, such as immunological and molecular ones. In Biology, morphology studies will remain the most straightforward approach to this hidden universe called microcosm. Morphology is a Queen that will always award her devotees! I just finished a long series of SEM shoots, it is above midnight here and I felt I have to defend my Queen. Yorgos Nikas eikonika@otenet.gr Tue Sep 15

Absolutely! Time is expensive! The time taken to treat the plants or animals, take samples, process them, etc. Imaging is cheap, and you don't want to have to go back and redo imaging because you didn't have enough samples to begin with. And sectioning doesn't take that long for an experienced person. Nor is it much more effort to run multiple samples. As an aside, in plant biology, it sometimes takes a year to get transgenics, yet people are often still unwilling to spend more than about a minute taking the key image showing the phenotype/gene expression. Another question is how many replicates are needed in experiments - for statistical differences to be detected? Also, how do you know what are the representative morphologies without multiple samples? Is any quantification needed? It often is, these days, you can't get away with saying "this is a representative image" so much anymore. And if you don't look closely at your tissues and cells, you might miss something subtle. Rosemary White rosemary.white@csiro.au Tue Sep 15

TEM:

tilting effect on camera constant

We have a mineralogy grad student who doing selected area electron diffraction (SAED) on single mineral grains and is collecting patterns while tilting the sample holder [X-tilt only between 0–20 degrees]. The student is careful and thorough and is worried that the tilt will change her camera constant. To what degree does stage tilt affect the camera constant, and is there a recognized way to compensate for the change? Any assistance, suggestion, or reference is appreciated. ***Tom Williams tomw@uidaho.edu Thu Oct 8***

The problem, as you rightly understand, is that a focal length change in the objective lens will change the camera constant. There are two ways to correct this type of problem—either set the eucentric point very accurately prior to tilting, or always focus the tilted specimen by using the eucentric height control (Z'). Either method is acceptable, but I would use the latter in preference. Steve Chapman protrain@emcourses.com Fri Oct 9

SEM:

30KV fails automatically

We are using a FEI Nova NanoSEM450. Whenever we use the 30 kV, it only last for around 5 hours before it fails. And if we turn on the beam again using 30 kV, it will turn off instantly again. But it allows us to use 30kV the next day, and of course still only 5 hours. The 20 kV and lower work fine. The technician checked everything but didn't find any problem. ***Jason 13qw9@queensu.ca Fri Sep 11***

We had a similar problem with a Philips CM200 that would arc at 200 kV after several hours. Still had the problem after replacing the entire emission chamber. It turned out to be a DC power supply that regulated the high voltage. A 15 volt DC supply would start losing voltage causing the high voltage tank to increase to 220 kV plus then arc. It took a rookie service engineer, Ken Hurst, to setup a laptop to read all of the DC power supplies to find the one causing the problem. David Hull drhull@zoominternet.net Fri Sep 11

EDX:

origin of X-rays 0-100 eV energy

A typical energy dispersive X-ray spectrum recorded using a TEM or SEM has peaks corresponding to characteristic X-ray energies, superimposed on a Bremsstrahlung background. I notice there is a zero peak followed by some background with reasonable intensity and it appears differently by different detector manufacture in terms of intensity. I understand that ionization process results in the characteristic X-ray peaks. But, what process or electron/matter interactions could be involved to give the X-rays at low energy up to 100eV? My detector registered X-ray counts there; it must have come from somewhere. I would be extremely grateful if you could give me some hints, or suggest some book chapters to read, or some papers to refer to. ***Zhaoxia Zhou z.zhou@lboro.ac.uk Wed Sep 9***

The fact that this zero peak appears different from detector to detector is your clue to the origin, a peak or peaks in this region is almost certainly an instrumental effect, and not x-rays. The various components in the detector and processing electronics generate some electronic noise which is digitized along with the true x-rays generated from the sample. Most pulse processors have a set of discriminators that are set on installation to minimize the amount of these effect, but cannot eliminate them entirely. This is true even if you have a recent "digital" pulse processor–there will still be some software settings that act to reduce the zero noise peak. Every manufacturer does this a bit differently hence the difference you see. If you can modify the discriminator setting on your unit try tweaking then a bit and you will see some interesting stuff. Proper discriminator settings are critical to getting good light element x-ray detection with proper peak shapes and peak positions. Jon McCarthy jjmccarthy@wisc.edu Wed Sep 9

MT

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