NetNotes

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Selected postings from the Microscopy Listserver from July 1, 2011 to August 31, 2011. Complete listings and subscription information can be obtained at http://www.microscopy.com. Postings may have been edited to conserve space or for clarity.

Specimen Preparation:
bacteria for SEM

I am having some difficulty with dehydration of my specimens, and help I could get would be much appreciated. I am working with a Gram-negative, motile, alpha-proteobacteria. My general protocol is as follows: 1) Fix for 15 min. at room temperature with 2.5% glutaraldehyde in a 0.1 M Sodium Cacodylate solution (pH ~7). 2) Dehydrate with an ethanol series—30%, 50%, 70%, 100% (×3), then critical point dry. Unfortunately, my bacteria are coming out wrinkled, shriveled, and just generally improperly dehydrated. I have tried several permutations of this protocol including fixing at in the fridge for an hour, post-fixing with 1% osmium tetroxide for 30 min. before the dehydration process, but I have not found anything that has really helped. Does anyone have advice/experience with this sort of preparation? ***Benjamin Brezler benjamin.brezler@gmail.com Mon Jul 11***

When I have problems, I buckle down and do what Dennis Kunkel does (Google his stuff to see beautiful microbes): Your fix is OK, wash well, postfix 1% OsO_4, wash with cacodylate again, then (I think this is the trick) dehydrate in ethanol, 10%, 20%, 30%, 50%, 70%, 85%, 95%, two times each dilution; first time for 5 min, second time for 15 min. Don't rush this part. When in 70% ethanol, transfer to filter holder (or smooth lens tissue origami packets). Dehydrate in 100% ethanol 3 × 10 min, then critical point dry. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Mon Jul 11

Your fix seems short to me—I generally use 30–60 minutes in 1% to 1.25% glut in buffer. The dehydration definitely needs expanding—especially the lack of 90% and 95% ethanol. What do you mean by "critical point dry"? That is, what is your exact procedure? You have to think of the CPD in the same way as you do the dehydration. The ethanol must be replaced within the cells with liquid CO_2, which requires flushing and soaking cycles, analogous to the dehydration steps used to substitute H_2O with ethanol. I generally use 5 × 5 minute soaks when doing bacteria on membranes. If I'm doing cells on agar, I treat them as tissue—the agar has to be dried properly to maintain the relationships between the cells (they're a community, not just a bunch of bugs). The time and number of steps varies with the cells, the community (how many different types, what kind of biofilm, etc.), and is pretty much empirical. Have you tried drying from HMDS (hexamethyldisilazane)? That can work very nicely. And some bacteria will air dry from 100% ethanol or acetone just fine. Phil Oshel oshel1pe@cmich.edu Tue Jul 12

I do an extended fixation in glutaraldehyde (1 day) and osmium (1–2 days), followed by dehydration in ethanol like Tina describes. Then, a long exchange of CO_2 in the CPD device, like Phil mentions. John J. Bozzola bozzola@siu.edu Tue Jul 12

Joining in the chorus of excellent responses you've gotten so far, here are a couple of thoughts: In my hands, short (<4 hrs @ room temperature) fixations of bacteria can cause later shrinkage problems. Admittedly I usually work with Gram-positives, but even Gram-negative bugs often need at least an overnight fix. If you're trying to maintain any extracellular matrix, this problem becomes even more acute—16–24 hrs at room temperature is where I usually start. Though not usually an issue with single bacterial cells, the osmolality of your fix is pretty low, too. As other commentators have noted, this ethanol series is short and pretty abrupt: I've moved toward more steps rather than fewer (I typically do eight gradations—25% → 50% → 70% → 85% → 95% × 2 → 100% × 2). Even this may not be enough: I'm considering moving to 10 stages (adding an early (~10%) and a late (~90%)) for my most morphologically important specimens. Tina also suggested lengthening the transition times—while it hasn't helped my specific preps, it is an excellent point. Overall, I think you may be trying to rush your preps. Good SEM prep takes lots of time (and figuring out the most efficient prep for your particular sample often takes even longer). Aaron Barnes barnesa@umn.edu Tue Jul 12

Specimen Preparation:
freezing glutaraldehyde

We routinely store small ampoules of our glutaraldehyde in the freezer. Someone noticed that 4 out of the 20 or so ampoules did not freeze even after 4 months. We first thought they must be different concentrations, but they are all 8%. Anyone know why this might occur? ***John J. Bozzola bozzola@siu.edu Wed Jul 20***

I do not have the answer but will share a similar observation with Mowiol mounting medium. I freeze 2 mL aliquots of a batch and store them all in one rack in my -20 C freezer. Invariably some freeze and others don't. It doesn't appear to be related to where they are stored in the freezer. Initially I blamed this on technicians who failed to adequately mix the ingredients. But then I made a batch and stirred it for a ridiculous amount of time and still observed the variability. Perhaps the answer to John's question will also solve my mystery. Tom Phillips phillipst@missouri.edu Wed Jul 20

Have you checked to see if you have a non-frosting freezer? Some freezers routinely switch on a thawing cycle to remove ice from the chamber. You can easily tell of you have one or not—if your freezer is filled with ice, you don't have one. If there is no ice build-up, it is because the machine is thawing it on a time schedule. Paul Webster pwebster@hei.org Wed Jul 20

This is a good sign! Obviously your solution in the state of a super-cooled fluid and is very clean (pure) and without any particles (serving as starters for freezing of ice crystals) just tap hardly on the vial immediately after taking from the freezer and usually it should freeze (get solid) within a second. Peter Heimann peter.heimann@uni-bielefeld.de Thu Jul 21

Specimen Preparation:
sodium ethoxide

I need to etch some Epon from my blocks, and I intended using sodium ethoxide. Unfortunately, I can't find the detailed protocol that

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I used several years ago. Can anyone provide a detailed protocol for the preparation? ***Josif Mircheski jmircheski@us.es Thu Jul 21***

I usually just dump a bunch of sodium hydroxide pellets into a glass Schott-style bottle and then add about 50–100 mL of ethanol to it. You want a saturated solution so if all the pellets dissolve, add more. You could use a stir bar to speed equilibration but I usually do it a couple of days beforehand and give the bottle a swirl once or twice a day and that is enough. It is obviously a caustic solution so be careful. Wear gloves and I usually require my students to wear safety glasses when using it. I simply carefully decant off the solution when I want to use it. Many anecdotal comments I have read on line over the years suggest it gets stronger the longer you let the solution sit. When I want to etch the sections, I use this procedure: (1) Pour about 40–50 mL of sodium ethoxide solution into a glass Coplin jar. This solution is very caustic—use gloves and eye protection! (2) Etch number and circle sections on the slides using a diamond pen. Rinse the slides with dH_2O. (3) Carefully insert slides into the caustic sodium ethoxide solution using a forceps. Leave in etching solution for 1 hour. (4) Fill 3 Coplin jars with 95% ethanol, 70% ethanol, and dH_2O. (5) Using a forceps, transfer the slides from the etching solution into the 95% ethanol for 10 min. (6) Using a forceps, transfer the slides from the 95% solution into the 70% ethanol for 10 min. (7) Using a forceps, transfer the slides to the distilled water Coplin jar. Transfer the Coplin jar to the sink and slowly run distilled water into the jar for 10 min. (8) Let the slides dry. Tom Phillips phillipst@missouri.edu Thu Jul 21

Microscopy:
errors in terminology

The latest Microscopy Today just bounced on my doorstep and I have to say I am 100% with Charles Lyman's editorial comment! And, whilst we are at it, what about destroying the other errors in understanding that have made their home in our subject? Most errors seem to crop up in SEM terminology. Take objective lens as a name for the third condenser, it does not follow microscopy parlance in that the does not produce the image, it simply acts as a third condenser. In truth it is the instrument's electronics that produces the image. Then there is depth of field when what people are talking about is depth of focus. How does the position of an imaging surface explain the way we visualize depth in an image? Please, please let us sort out depth of field and depth of focus too! Microscopy has an amazing history, so abusing names that were part of that history does seem so wrong! ***Steve Chapman protrain@emcourses.com Fri Jul 22***

I agree that the terms "depth of field" and "depth of focus" are too frequently interchanged, but I feel that you, too, have fallen into the same trap. These two terms are well-documented and describe two quite different features in an imaging system. You got it correct in your 1986 book: Working with the Scanning Electron Microscope (page 22). Depth of field relates to the object space and depth of focus relates to the image space. Some microscopists use "depth of focus" when they really mean "depth of field"; the confusion seems to come from the fact that as the focus control is adjusted, different parts of the specimen (in the z-direction) go in and out of focus. So, when photographers (who first coined the terms) and light and electron microscopists use the term "depth of field" they are correct if they are talking about how much of the subject or specimen is in acceptable focus. Depth of focus is used to describe the how much a camera can be moved from the film /detector plane before the image becomes unacceptably out of focus. Depth of focus is usually a fixed distance that cannot be changed because it is unusual for the microscopist to be able to move the film or detector plane away from the imaging/projection lens. For digital imaging in an SEM, depth of focus is a redundant term because there is no camera, so a microscopist shouldn't even need to think about this term nowadays. Microscopy does have an amazing history and the two terms are well-established and easily understood, so any attempt to change their meaning will result in even more confusion. I hope that the depth of field and depth of focus is now sorted! Gary Nichols gary.nichols@pfizer.com Mon Jul 25

Thank you for pointing out a misunderstanding I may have portrayed. When using the term "imaging surface" I was referring to the "film plane." For those whom I may have confused, I think the TEM portrays depth of field and depth of focus very clearly. It is easy to see how small a depth of field the operator has (just microns) and it is clear the depth of focus is almost limitless with an image in focus on a digital camera both above and below the screen! Steve Chapman protrain@emcourses.com Mon Jul 25

"Microns" of depth of field was a long time before aberration correctors became popular. I think this discussion of depth of field will become more and more important as correctors allow larger convergence angles and subsequently severely reduce the depth of field. Furthermore the effect of sample thickness, convergence angle and also channeling will become more and more important especially due to the popular pass-time of analyzing at the atomic level directly down a zone-axis. In terms of systems with probe correctors, the depth of field goes to a number in the nanometer range. Well perhaps a few tens of nanometers. Jan Ringnalda jan.ringnalda@fei.com Mon Jul 25

This is of course very interesting. But I have a totally different experience that was and has been exasperating. Filing the Patent and Trademark Office Form VA for copyright registration of SEM, LM and TEM pictures, the application was rejected . . . . not once but several times. VA is visual arts—photographs. Well, the examiner rejected the application the first time because an image from a SEM or TEM was not a photograph. In his view, it was the product of a method of producing images and hence, was a patent issue. Sigh. There was no optical glass lens. There was no film (for SEM) but a negative for TEM. The explanation I gave about how a TEM and SEM "images" were formed based on scanned or transmitted electrons did not connect with visual arts. Too technical of an explanation of how SEMs and TEMs work—oops. Electron optics was a deal killer. Re-submitted and hope to get a different person. Nope. OK. Change the submission title. Re-submit. Now waiting for several months. Patents are backed-up too as I hear. An objective lens made from copper wire? That did not fly. No shutter or flap mirror. No viewing prism. Registration denied. Finally, sanity should prevail. Gary Gaugler gary@gaugler.com Wed Jul 27

Instrumentation:
sputter coater

I am attempting to remedy an issue with a Conductavac IV sputter coater by SeeVac. Recently, it was observed that the ammeter needle was wavering considerably during the sputter coating process. The needle would be at 0 mA and with a slight twist of the Current Adjust Knob the needle would be up near 40 mA (~ half way up ammeter). I assumed there might be a short somewhere in the system and after checking all the connections and making sure all junctions were snug no obvious problems were observed. Another sputter coating run was attempted but to no avail. However, the needle now moves with more fluidity. Control of the needle has been regained though the needle currently maxes out (100 mA) where the needle would typically be at 30 mA (proper amps for general sputter coating). Why the rapid increase in amps? I have also

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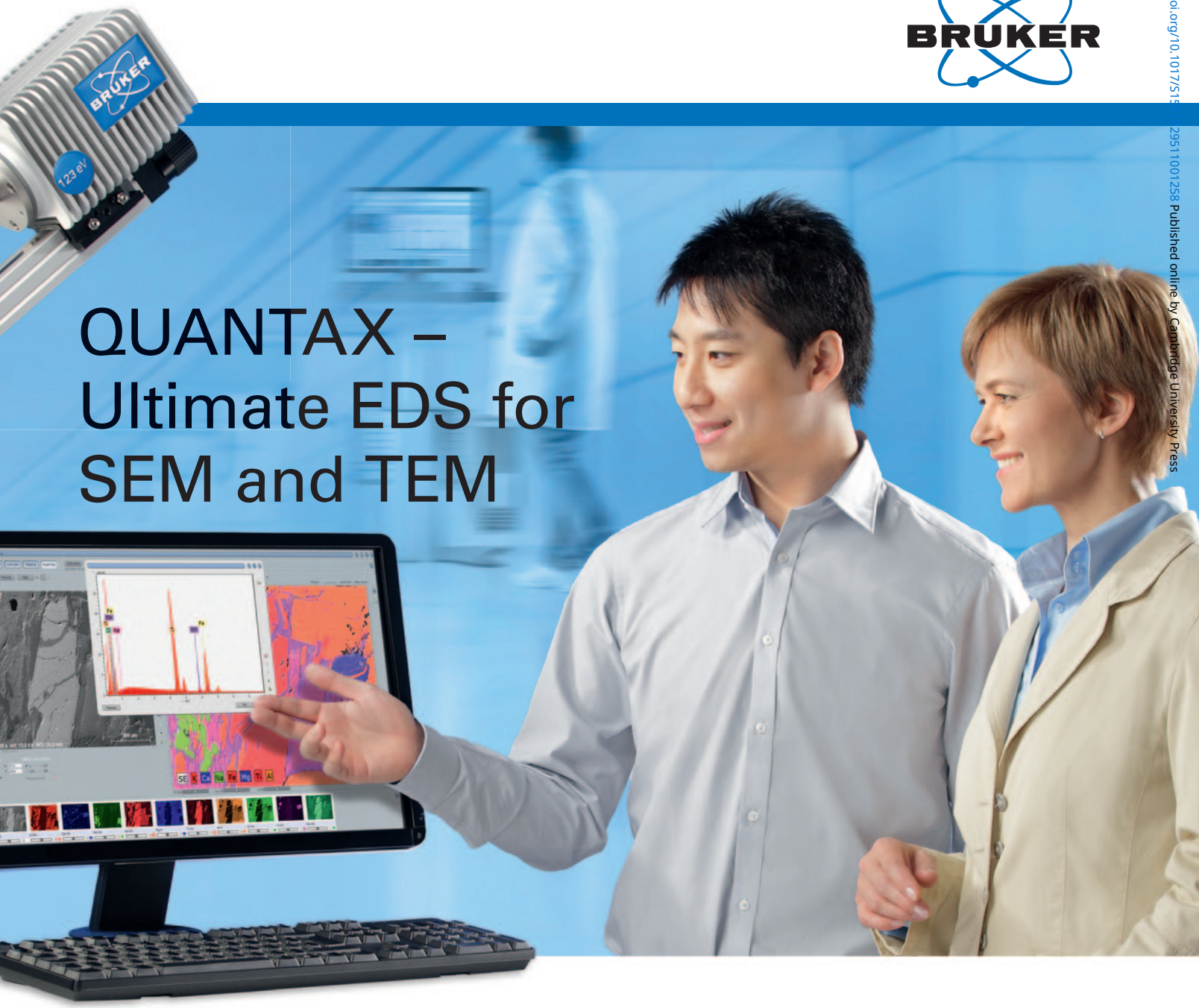

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cleaned the anode and cathode hoping the removing any debris would help solve my issue. The vacuum pumping out the system is working fine and easily pumps below 100 mTorr. SeeVac appears to have gone under or been bought since making the Conductavac IV (~25 years of age) so trying to find a troubleshooting source has been difficult. Any suggestions, whether is specially pertains to the Conductavac IV or any sputter coater, on my amp problem will be greatly appreciated. ***Joe Heintz josephheintz@gmail.com Thu Aug 4***

Check for breakdown of the high-temperature epoxy around the electrical feed-through at the top. I had that problem in the past. Phil Oshel oshel1pe@cmich.edu Thu Aug 4

My start at troubleshooting would be to determine (1) if the ammeter is at fault or (2) if the current being measured is really fluctuating as the meter seems to indicate. Background: Ammeters are typically voltmeters that measure the voltage drop across a shunt resistor. Then, using Ohms law, the current measured = I = V/R, where V is the voltage drop measured and R is the value of the shunt resistance. The shunt resistor may be external to the meter, inside the meter case, or for very low currents the resistance of the meter coil itself will suffice (there would be no shunt resistor—external or internal—in this instance). Suggested troubleshooting: After rechecking and cleaning any connections to a possible external shunt resistor, I would substitute a good digital volt-ohm-meter (a typical Fluke 112, etc.) for the suspect ammeter, set to read amperage, and see if the fluctuations reproduce. With the results of this test, you should then know whether the meter needs replacing or you need to keep looking elsewhere. Rick Ross richard.ross@allisontransmission.com Thu Aug 4

Instrumentation:
cleaning mirrors

I'd like to know if anyone has attempted to successfully clean the oil off of a dirty mirror without ruining it or drilled out a larger hole through the mirror to get a wider field of view. Please let me know if you have tried these things, even if it didn't work. ***Dan Ruscitto ruscid2@rpi.edu Thu Jul 14***

I routinely clean dielectric-coated front surface mirrors in positioning interferometers by blowing dust off and solvent/blow dry cleaning with airbrush. This method may work for metal front-surface mirrors, but I never tried it. Instead of drilling larger hole in existing mirror and risking ruining the instrument, I'd suggest making a replacement with the desired hole and substituting it for existing. Higher cost, but you have a solid fallback plan in case something goes wrong. Valery Ray vray@partbeamsystech.com Thu Jul 14

Instrumentation:
Lytro camera

Has anyone else seen this: http://www.lytro.com/? My immediate questions: 1. Does it actually work? 2. Can it be applied to microscopy? ***James M. Ehrman jehrman@mta.ca Fri Aug 12***

Is it just a fancy way of presenting extended focus images? You click on the picture and it knows which image in the through focus series to show? Rosemary White rosemary.white@csiro.au Sat Aug 13

This type of camera is known as a plenoptic camera. Wikipedia has a short description of this technology here: http://en.wikipedia.org/wiki/Plenoptic_camera the references do a good job explaining the technology in depth. In fact, reference 2 is a paper coauthored by the founder, Dr. Ren Ng, of Lytro. This video from Adobe shows the technology in action: http://www.youtube.com/watch?v=jS7usnHmNZ0. Chris Winkler microwink@gmail.com Sat Aug 13

I saw this new technology, and my first impulse was the same: Can that be used for microscopy? If you look at the technology, it works essentially by breaking up the image into many smaller images through the use of an array of microlenses. These lenses are spatially distributed, and each one sees the object from a slightly different angle. A modern light microscope is "infinity corrected." The objective lenses are designed so that the light is focused to infinity. If I understand that correctly, the microlenses in such a camera would all see the same image, and the images could not be focused after the fact. Of course, it might be possible to design objective lenses that allow the use of such a camera. Mike Bode mike.bode@resaltatech.com Sun Aug 14

Instrumentation:
inverted microscopes

I am a big fan of data, calculations or references if you can supply any. There is some lore in my facility that inverted microscopes are optically inferior to upright microscopes. I realize they have a more complicated light path. Therefore, I'm wondering with a modern microscope how inferior, if at all, are inverted microscopes compared to upright microscopes everything else being equal (objectives, filters, camera and sample preparation). ***Mary Raven mary.raven@lifesci.ucsb.edu Wed Aug 17***

The customary difference is that an inverted scope is supplied with a long working distance and hence lower NA condenser. So for transmitted light modes, the NA of the inverted condenser is too small to fill the objective and hence imaging suffers. Note that this doesn't matter for fluorescence and other incident light modes, and many inverteds can be equipped with a high NA condenser and thus brought to par (of course the working distance will go down). Tobias Baskin baskin@bio.umass.edu Wed Aug 17

TEM:
biosample at 200 KV

We have one user that prepares his biological tissues in resin and then cut them for TEM studies. He has been using an 80 kV TEM for that, but that TEM doesn't have digital camera and he wants to start using ours. We have a JEOL 2100 TEM operating at 200 kV. We can run the sample and do some imaging while playing a bit with the settings of the scope, but still in some cases the sample ends up burning. I'm not experienced with biological samples. Is there a different sample preparation he should look for, or maybe change his resin or do cryo-cut, before coming to our scope, or is there a certain group of settings I should work with for this particular case? ***Marcela Redigolo marcela.redigolo@mail.wvu.edu Wed Jul 27***

By burning do you mean bubbling or exploding sections? Sometimes a thin carbon coating of the section will improve things greatly, even if the sections are on carbon-coated Formvar or lacey film supports. I can use a 10 nm STEM probe in a 2100F for EDS line scans and maps of biological sections—if carbon coated. Without the carbon coating the films can explode under the beam. In TEM mode I tend to use spot sizes 3–5 to minimize beam exposure/damage. I do not believe a cryo cut would be of any value. A small objective aperture will help prevent/balance charging effects. How thick are the sections? I generally find 150–250 nm quite fine. I grew up in the Materials world and now manage a structural biology TEM facility. HAADF STEM can be a great way to image biological low contrast samples. Roseann Csencsits rcsencsits@lbl.gov Wed Jul 27

Your 200 kV microscope will work very well down at 80 kV and when you return to 200 kV it will regain its absolute stability

quite quickly. I had a similar incident with a client who also worried about changing from their 200 kV JEOL. I had the client take test pictures of the gold lattice from 200 kV down to 80 kV in 20 kV steps; she was 100% successful! Steve Chapman protrain@emcourses.com Thu Jul 28

Just to add what may be an obvious note about operating at a lower accelerating voltage. If the 2100 has never been aligned at anything other than 200 kV, you may need to have a service engineer in to do initial alignments at lower voltages. When our JEOL JEM-3010 was installed, the engineers did alignments at 50 kV intervals from 50 to 300 kV and stored those values for future use. On the rare occasions when I use a lower accelerating voltage, I just have to do some minor touch-up to the alignment. Also, you'll want to check your magnification calibration at the lower accelerating voltage. Elaine F. Schumacher eschumacher@mccrone.com Thu Jul 28

I also needed to adapt biological sections for use in a 200 kV TEM. I routinely purchase carbon/Formvar-coated copper grids (200 mesh) and collect the sections on the 'light' non-carbon side of the grid (less phobic and easier to collect sections from the boat water). The stability of the carbon plus support film permits the use of thinner sections (less than 70 nm) and there is less drift of the material under the electron beam. The electron beam can be focused to a fine spot on these grids for making height adjustment and for x-ray analysis. I did not like carbon-only coated grids because of the increased background texture. I stopped trying to make these grids myself—the time involved and inconsistent quality of my homemade grids was not worth it. The increased grid cost is offset by your reduced photography cost. Changing kV to 120 in the instrument required additional magnification calibrations and although this may improve contrast in the biological material on the screen, the digital camera we use provides excellent contrast in the final image. So depending on the camera and your final images, a lower kV may improve contrast but I would definitely recommend carbon/Formvar-coated grids. Susan Belfry belfry@unb.ca Thu Jul 28

Just one additional thought: did you insert the objective aperture? Perhaps on the 200 kV (EDX?) you mainly work with the aperture out. It needs to be inserted otherwise there is a big chance that you blow up your section. Stephane Nizets nizets2@yahoo.com Wed Aug 3

SEM:

ion pump problem

We are currently experiencing a problem with our Carl-Zeiss EVO 40 SEM. We have fitted this SEM with a LaB_6 filament, and the gun area is pumped with an ion pump. We occasionally (every 3–4 months) bake the gun area to reach ~2–3 × 10^{-7} mbar vacuum levels as a requirement for the LaB_6 filament operation. During the last bake, we found that the ion pump stopped working during the cool down stage while the final vacuum level in the system was good around 10^{-7} mbar levels. However, since then we were not able to keep the ion-pump working. When we turn the ion pump on, it slowly dies away. That is, the reading of the vacuum level in the gun area is thru the ion current (there is not an extra ion gauge in the system), and the vacuum level starts at 10^{-8} mbars and within 5 min goes down to 10^{-11} mbars. Finally, the ion pump shuts down. This pattern repeated after several restarts of the system. I tried gently tapping the ion pump with a plastic handle of a screwdriver, which kept vacuum level around 10^{-8} and the pump running, but I think that is not a viable option for running any SEM with decent resolution. ***Erman Bengu bengu@fen.bilkent.edu.tr Wed Jul 20***

Ion pumps have a long, but limited lifetime. Most likely internals of your pump became coated with dielectric deposits, and you will need to replace it. If new pump is beyond the budget, then you can send your original pump to be rebuilt. vray@partbeamsystech.com Wed Jul 20

Gentle taps with a screwdriver? I can remember the Phillips engineers hitting the magnet of the ion pump on the Bristol EM400 with a 2″ by 4″ block of wood. This was to knock the whiskers off the inside of the pump which tended to cause it to short out and not operate. If you are going to have to replace the pump anyway, a couple of fairly brutal knocks might be in order, just to see if you can eke out a couple more months. A rebuild is probably in the offing, but you might be able to stretch the life of the current one. John Mansfield jfmjfm@umich.edu Wed Jul 20

Use a hammer and pound it. If you are doing it right, you should also use hearing protection and eye protection. John Mardinly John.Mardinly@asu.edu Wed Jul 20

Dr. Mardinly's comments about using a hammer to dislodge whiskers inside an ion pump leads me to point out that if the whiskers are not too firmly established it is often possible to get rid of them by turning the high voltage supply on for a few seconds (not too long or you may damage the power supply) three or four times with the pressure in the pump above 1 Pa (10^{-2} Torr), If this approach works it is gentler, and quieter, than the hammer method. Incidentally, this method (but not the hammer method) and other characteristics of ion pumps, are described on page 295 of my book, *Vacuum Methods in Electron Microscopy*. Wilbur C. Bigelow bigelow@umich.edu Wed Jul 20

This is the correct procedure how to remove whiskers, however I do not think that Erman's problem is caused by whiskers in the ion pump. Presence of whiskers causes additional field emission current and as a result it appears like additional (leakage) current. Such ion pump shows higher ion current and therefore it measures higher pressure than expected. Erman's ion pump shows unexpectedly low pressure. Therefore, it could be a problem of the ion pump controller (e.g., lower voltage than usual); ion pump is not necessarily bad. I would not use a hammer in this case. Tomas Hrncir tomas.hrncir@tescan.cz Thu Jul 21

SEM:

Epon blocks

Has anyone examined Epon blocks in a SEM? I tried to image my muscle samples embedded in Epon with a SEM. (the same block was previously used for ultra-thin sections, so its surface was pretty flat). The only thing I could see were brighter than background spots representing the muscle fibers. I'll try to remove some resin from the surface with sodium ethoxide, so that only the sample "sticks out" a little bit over the resin, unembedded. Has anyone else done something similar (seeing Epon blocks in SEM)? Could you please share your experience? ***Josif Mircheski jmircheski@us.es Thu Jul 21***

I haven't seen any replies to your question on the listserver. Yes, I have done SEM on Epon thick sections without etching them, which work better than using the block. Use a razor blade and hand-trim a "thick" section (actually as thin as you can trim manually) from an Epon block after you have cut some thin sections from the block face to smooth the face off, and mount this section with the smooth side facing up on some double-sided carbon tape. Use a piece of plastic wrap (Saran Wrap, sandwich wrap, plastic bag) to press the section onto the tape. If you have access to a carbon coater, give the sample a light carbon coating to reduce charging. Put a couple of marks on the tape so you can find the section easily in the SEM, either by pin pricks or dots of silver paint. Now observe your section using your backscattered electron detector.

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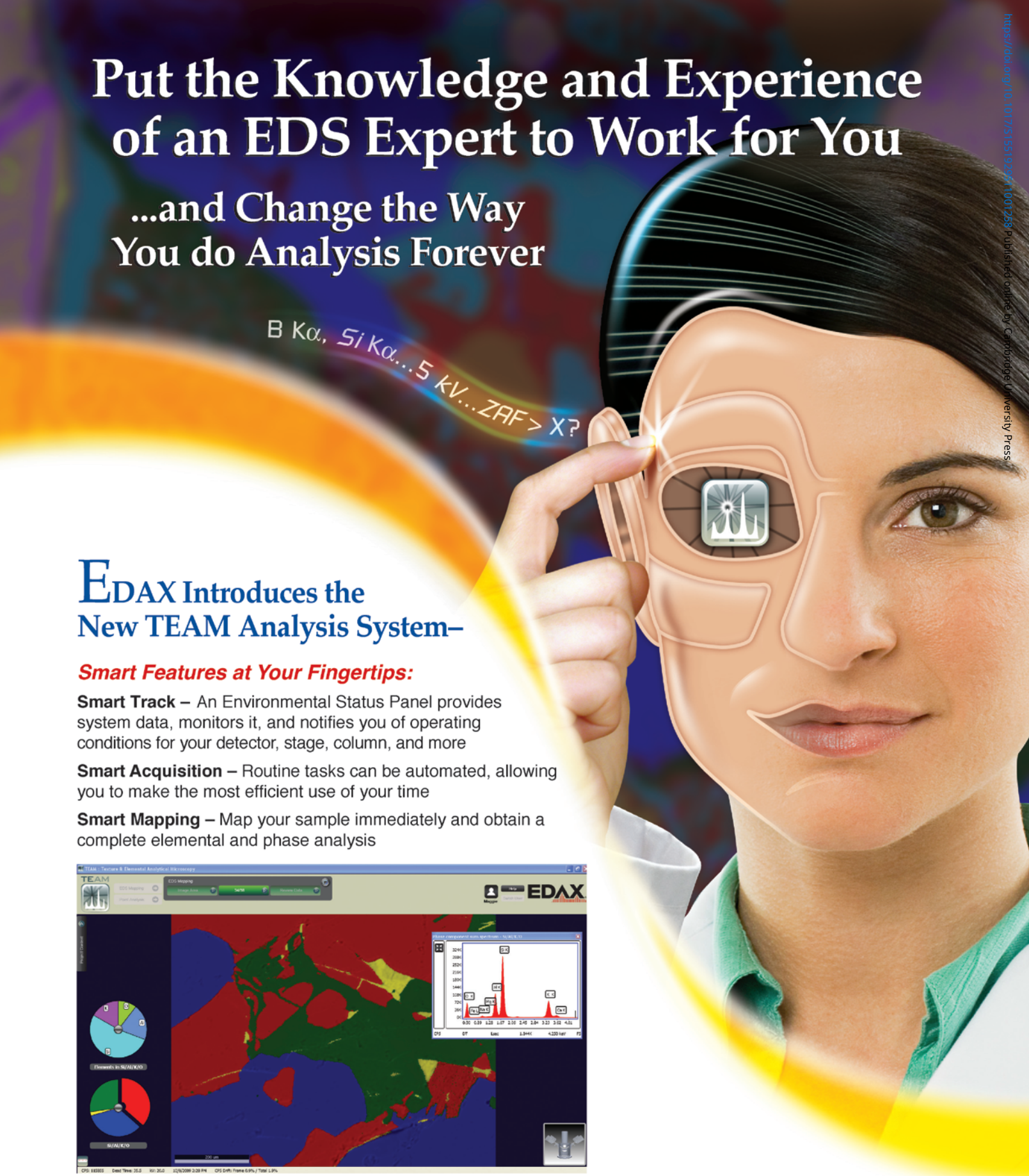

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I've never tried this with en-bloc uranyl acetate stained tissue, only osmicated tissue, but have been able to obtain photos of the tissue in this manner, and if the tissue had foreign particles in it, have located them rapidly by this technique for x-ray analysis. You may try staining your tissue with uranyl acetate and lead citrate and see if this is a possibility. I have not experimented with this. Ed Haller ehaller@health.usf.edu Thu Jul 21

Thank you very much for your advice and help. I tried to image Epon block faces, but without success. We used two microscopes, a JEOL 6460LV and a higher resolution Hitachi S5200. There were no environmental SEMs, nor a SEM with a microtome, available. We used coated and uncoated blocks, combined with etched and unetched resin. The result was always the same—zero, or close to zero. Now, after re-reading your messages, I see that many of you (Derrick, Zack, Ed . . .) suggest coating with carbon, and we coated with gold. Two reasons for our choice of gold: the carbon coater is broken, and the SEM technician has little experience in biological samples (I have zero experience in SEM) and assumed that it will be fine with the gold. Well, now I have to wait until September for people to come back from vacations, and don't know till when until the carbon coater is repaired. Then maybe I can tell you if it was successful. The question: what do you think, was the gold the reason that we could not see any fibers at the block surface? Will carbon alleviate the situation? And one additional, maybe worthy of another topic: the intention of the "Epon in SEM" was to check these blocks under SEM, so that we can use them later in a FIB/SEM. Is there anyone experienced in FIB/SEM? If so, have you ever tried to use Epon blocks/sections? Josif Mircheski jmircheski@us.es Fri Jul 29

You can attempt to view your block using a low accelerating voltage and not coating the block if you have a field-emission SEM. With the Hitachi S5200, you might be able to image at a few kV in backscattered mode uncoated and not melt the epon. It is worth a try. As soon as you coat with gold, you will cover everything in the block, and will see nothing inside the block unless it has a higher atomic number than the gold itself, or unless it is physically sticking out of the surface of the block. With the FIB, although I don't use one, you may be able to preferentially etch away the epon to get some surface relief of the tissue, since the tissue is osmium fixed, it may be slightly harder than the plastic, and may resist etching a bit. I haven't tried this. If that's the case, then you could lightly coat with gold. You might have to explore etching at different angles to do this. Edward Haller ehaller@health.usf.edu Fri Jul 29

SEM:
filament heating current

Our SEM JEOL JSM-5600LV is experiencing some problems in the filament heating current (thermoionic emission, W hairpin filament). At first the current was very unstable, fluctuating between about 15 and 150 μA. Now it has more or less stabilized, but at values definitely too high. I can try to clean the gun components, at least, but I've never opened an SEM gun. In particular, I wonder if there's some isolating gas like SF_6 in TEM guns. Does anyone have advices for me? ***Davide Cristofori dcristofori@unive.it Mon Aug 1***

Opening the gun is easy, and there should be full directions in the SEM manual. Your symptoms sound like most like either the filament has physically drifted and needs to be (manually) recentered, or it has grown a whisker that is approaching the Wehnelt. If you are very careful, the whisker can be knocked off without disturbing the filament. Just make sure the now loose whisker is not rattling around in the Wehnelt, then reinstall the Wehnelt and you should be OK. But, expect to have to replace the filament if it is a whisker. Philip Oshel oshel1pe@cmich.edu Mon Aug 1

I forgot to say I checked the filament and no whiskers are present. Indeed, the problem is present also with a couple of brand new filaments, from different boxes (but not sure they're from different batches). Also the filament position looks the right one, and actually the hardware doesn't allow for such big drifts. As for opening the gun, I mean "dismantle," because the idea was to check for some problem on electrical contacts for the heating current and high tension. Just an attempt, because we had a similar problem with TEM time ago. So, is it safe for the gun to dismantle it without knowing how the instrument is built? May there be SF_6 or any other insulating gas? Davide Cristofori dcristofori@unive.it Mon Aug 1

I'm assuming the gun is of similar construction to the 5200, 5300, 5400. There is no gas. There are pieces of red silicone rubber and a machined nylon core. This can all be disassembled, cleaned, inspected carefully for arc tracks and reassembled with a small amount of silicone grease on the silicone rubber parts. It is a smaller version of the 840/6400 gun. I have had to get a new nylon piece machined for a 6400 that had arced through due to mostly being operated at 40 kV. I also had a 5200 with a bad cable which I tried repairing/replacing several times but had to finally give up and by the cable/gun assembly from JEOL. They would only sell the complete assembly. Before you go through all of that, is the gun vacuum good enough? Having the emission current bounce all around could possibly be arcing in the gun due to poor vacuum. The arcing could also have damaged components in the HT tank. Check the bias circuit carefully. Ken Converse kenconverse@qualityimages.biz Mon Aug 1

SEM:
colorizing images

I am looking for the best way to color SEM images. I am only aware of a few techniques such as (a) acquiring images at different accelerating voltages and using them as RGB channels, (b) using Matlab scripts such as "Colorization using Optimization" or (c) using custom color tables. However, none of these techniques gives me the stunning images I have seen from various groups. How are the stunning color SEM images generated? Should I use Adobe Photoshop? I have access to a regular SEM with one SE detector and one BSE detector. ***Ram Tiruvalam rct204@gmail.com Thu Aug 11***

A simple way is to take 3 separate images with individual quadrants of your BSE detector, then combine them in Photoshop or other package, coloring the individual channels red, green and blue, respectively. Or some other color combination. Someone else will probably know the software package you can buy to do the other type of image colorization—where individual objects are colored differently. Rosemary White rosemary.white@csiro.au Thu Aug 11

Do you mean colorized like my images http://www5.pbrc.hawaii.edu/microangela/ or Dennis Kunkel's http://www.denniskunkel.com/ all taken with "my" SEMs at the University of Hawaii? We use Photoshop. In short, you need to learn to use your selection tools (especially Magic Wand and Lasso) really well, then save and use the selected areas as masks so that you can paint or fill with color in Color Mode. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Thu Aug 11

As others from the list answered there are two principal ways to get color in your SEM images: make good black and white images and add a lot of "Photoshop magic" to bring color to the specimen—use multiple detectors and perhaps also different kind of detectors (like SE or backscattered) to get different signals back from the specimen and use these signals to attribute aesthetical colors to the image (and add some Photoshop magic). For example: I use the secondary

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electrons for getting a very fast separation of parts on specimen with a small interaction volume like hairs on insects or cells or substrate etc. The backscattered detectors (I have three) I use to get surfaces (topography) ad hoc separated in a different color. You can also try to get more different images with different accelerating voltages and the best suited detectors for it... Also distance of the detectors to the specimen can make very different images. There is a PDF from a Mikrokosmos article which describes my work, but only in the German language: www.elektronenmikroskopie.info/pdf/Mikrokosmos06-99.pdf (1 MB) I am using a digital image acquisition system from point electronic, www.pointelectronic.de, which can handle up to 8 detectors and scan up to 4 detectors in a resolution of maximum 16K × 16K pixels. The scanning software has the ability to directly mix the signals from the SEM into a colored image. For the images, have a look at: http://www.elektronenmikroskopie.info/galerie%20-%20rem-biologie.htm for biologic specimen and http://www.elektronenmikroskopie.info/galerie%20-%20rem-material.htm for some images from material science. Sorry, pages are only in German language up to now and the site is still in construction. Stefan Diller stefan.diller@t-online.de Fri Aug 12

EDX:

nanoporous material

I am doing some SEM/EDX analysis on nanoporous alloys containing Ag-Au-Pt with a pore size between 3–15 nm. This nanoporous alloy is on top of an alloy with the same elements. The average thickness of this nanoporous layer is around 8 µm. Typically, I prepare metallographic specimens to either determine the thickness of my nanoporous alloys or to analyze its composition by doing EDX on a cross section of the sample. However, I found that because the Pt concentration is very low (~1 at.%) I have to use accelerating voltages higher than 20 kV. I have tried lower accelerating voltages obtaining very weird EDX results. I have two main concerns. One is about the effect of porosity in my results because I know that this type of analysis assumes 100% density whether you are using standardless routines or with standards. My second concern, which is related to the first one, is if there is any real concern of having an interference of the substrate in my EDX reading, mainly due to high interaction volume and high porosity. Therefore, I was wondering of there is any correction or consideration to take into account to do this kind of analysis. ***Adrian Vega adrian.vega@utoronto.ca Wed Aug 10***

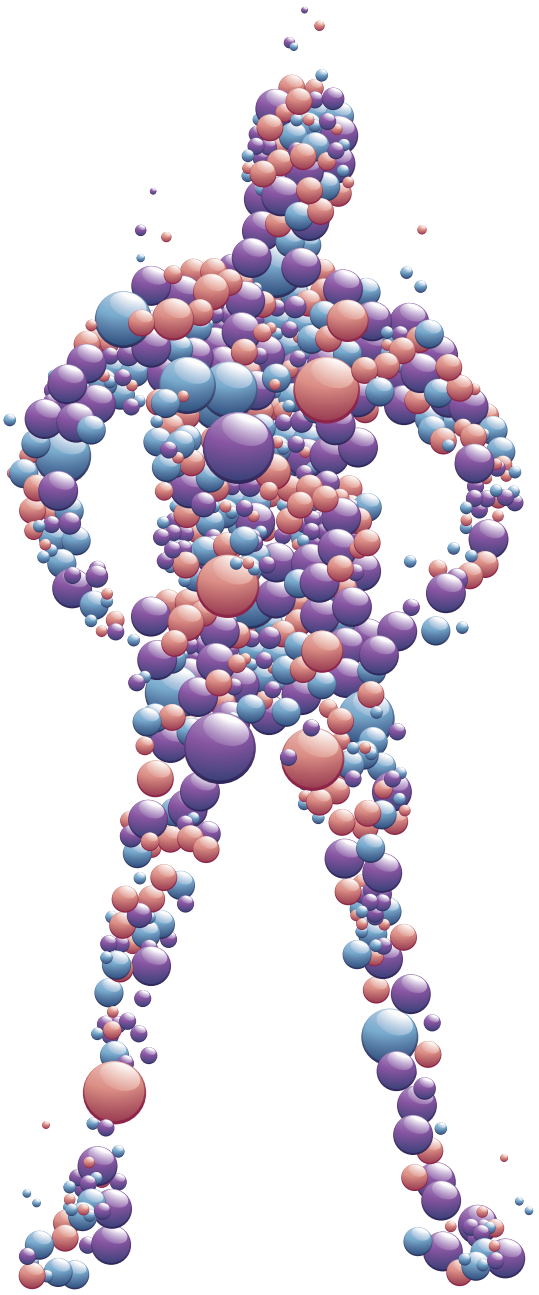
Given those elements, you may be okay using higher voltages. You probably want to use something like Casino or some other Monte Carlo method to estimate the excitation volume. I ran simulations on pure Ag (the lightest component) and found that the excitation depth was about 1 µm at 30 kV. It was about 700 nm at 25 kV and about 500 nm at 20 kV. You should not be exciting your substrate. I am uncertain what you mean when you say you had problems with low Pt concentration calling for higher accelerating voltages. What is your Au content? If it is high, then you need to make sure that your system is deconvoluting well. It will be more challenging to pick out a small intensity for the Pt M line next to a strong intensity for the Au line; however, it should be possible. You could use the L lines around 9.5 keV, but the intensity is much lower than for the M lines. Such challenging deconvolutions will need critical review rather than simply believing the numbers. You should check the fit and examine the residuals to make sure everything is accounted for. I recommend that for all critical applications. If there is a problem with your energy calibration and the peaks shift, then you could really have problems with the Pt intensity. Residuals would show leftover intensity on one side of the peak and a deficit of intensity on the other. Of course, that could be reflected in errors in the Pt concentration. You could also have problems if the stored peak widths are not the same as the operating conditions at the time of analysis. We have an old Oxford ISIS system. The factory peak profiles were collected at the highest resolution setting, but we usually reduce the pulse processing time to count faster. Our peaks are a bit broader and the default profiles don't fit the actual spectra very well. New software probably takes the pulse processing time into account and adjusts the profiles. Therefore, we have collected our own profiles under the exact same conditions. The default profiles are pretty good, but our own profiles take all possible differences between our system and the factory into account. I would recommend comparing results for L lines and M lines. At 30 kV, they should give you similar numbers if the deconvolution is good. I would also recommend testing the analyses on a sample with no Pt (maybe on a sample of pure gold). You could overlay the two spectra to see if Pt appears on a shoulder of the Au peak. You could also check the deconvolution results. If the software still reports Pt in the no-Pt sample, then you should downgrade the trust in your software. Offhand, I don't know that porosity of 3–15 nm should cause problems with the analysis, provided it is homogeneously distributed. It would allow a deeper penetration of the beam and it would allow a longer path-length for absorption on the way out. The mass absorption effects on the way in and the way out should be the same. EDS can be quite a powerful technique if done well with good software. Just beware that not all software is created equal. You will need to watch it carefully as you apply it to a number of problems and determine how much trust you can put into the results. Warren Straszheim wesaia@iastate.edu Wed Aug 10

Specimen Preparation:

precipitate in TEM

Can anyone suggest solutions (pun probably intended) to the problem of mystery precipitate on cells prepared for TEM? This is 5 nm–15 nm diameter black dots that appear on cells, but not on resin sections or on support film. The dots localize strongly to chloroplasts and myelin when osmium is involved, but not otherwise. In glutaraldehyde-only fixes the dots stay on the outside of the cells or in vacuoles. Many people have posted before about precipitates; on the basis of having gone through the Listserver archives we decided to try a series of experiments to try and get rid of the mystery 5 nm dots currently gracing all cells prepared for TEM in this lab. Our results are inconclusive, thus we are wondering if anyone else has tried anything and found a way of making the dots disappear. I have tried many combinations and permutations of local/ bought water, 3 different purities of glutaraldehyde, paraformaldehyde with and without glut, new osmium, lots of different buffers, new glassware, and plastic containers instead of glassware . . . the only thing I haven't tried yet is the ruthenium/veronal acetate fixes . . . Anyone with time to have a look can find details of some of the experiments in the pdf here, ⟨http://dl.dropbox.com/u/10613310/PrecipLIST-12May2011.pdf⟩ and these are representative of other results with different buffers. Obviously the cells aren't particularly nicely fixed - that's another set of experiments. Any suggestions most gratefully received. ***Giselle Walker giselle.walker@anatomy.otago.ac.nz Thu May 12***

I got similar precipitate when I didn't wash out the PBS enough before block-contrasting in uranyl acetate. Also, I got this type of precipitate when I left my samples on osmium too long (>3 hr, and they were not the ones with the PBS not washed) If you mix PBS (or any other phosphate) with uranyl, you will get fine precipitate. I am not sure about the mechanism of precipitate forming after longer osmium exposure. I read one paper where the author identified intracellular



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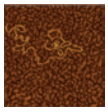

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calcium as black precipitate, but concentrated in mitochondria and not diffused. But I don't remember the paper, nor the author, and I am not sure how accurate is that identification. Josif Mircheski jmircheski@us.es Thu May 12

The dreaded black dot plague! I've struggled with this precipitate here in Tampa for years. It seems that our groundwater in Florida is rich in phosphates, and that the Millipore and similar filtration units don't always take all of the phosphates out of the water when the systems filter the water. This results in phosphate microcrystal precipitates in tissue processed for TEM if I use filtered water. When I switched to true distilled water, this issue resolved itself. I now use true distilled water to make my reagents in, and for my rinses. I work with phosphate buffered fixatives, which preserve the cytosol of mammalian tissue better. After glutaraldehyde fixation, I do a half hour buffer rinse, 3 × 10 minutes each, minimum, before osmication, and even longer if I am processing tissue such as myelinated nerve, in which case I rinse overnight in buffer. After osmication, I rinse in distilled water, again 3 × 10 minutes each, minimum, and longer if the tissue is thick or dense, to remove both the osmium and the phosphate salts. This has cured the precipitate problems for me. Some advice I gave another E.M. technician was to rinse in Tris buffer after glutaraldehyde and osmicate in Tris. The two buffer systems are compatible. You could consider this, also. Let me know what you find out. If you switch to cacodylate buffer, you should not get this precipitate, if phosphate salts are the culprit. I don't know if this is a possibility for you. I don't like the look of my tissue when working with cacodylate buffer, and the arsenic in the buffer is something I try to avoid. Ed Haller ehaller@health.usf.edu Thu May 12

Electron microscopy and electron probe analysis of mitochondrial cation accumulation in smooth muscle. Somlyo et al. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2109306/pdf/723.pdf. Perhaps this? Frederick C. Monson fmonson@wcupa.edu Thu May 12

Specimen Preparation:

microtoming material specimens

I have a student who wants to microtome some materials samples. The samples are GaSb and Si wafers. The length and the width are less than 1 cm each. The thickness, which is the most important, is less than 0.5 mm for GaSb and 1 mm for Si. The structures are on top of the support surfaces (same material). The GaSb samples are really not very hard. My concern is that these are students who have never touched a microtome. They need advise as to type of microtome knife to purchase and any other hints you have for success with these samples. Will it be necessary to embed them in resin (which one?) to stabilize the samples in the chuck? Most likely the resin will peel away from the cut sections but that is not a problem. I will teach them using a resin block and glass knives so that they get the idea but that is a long way from success with their samples. They are hoping for thicknesses of 100–300 nm and will be imaging them with a 300 kV FEI Titan TEM. FIB is not an option with these samples as the ion beam destroys the detail of interest. ***Debby Sherman dsherman@purdue.edu Mon Jun 13***

It has been done with semiconductors, but the results give you a bunch of cleaved stuff that doesn't look great. If they are looking for a relatively straightforward process for making TEM samples that doesn't require very expensive equipment, I highly recommend the MicroCleave(TM) technique, also known as the Small Angle Cleavage Technique. This technique is fairly easy to learn and it will make absolutely fantastic samples with these types of materials. Here are several links to application notes on our website. We also have a video on how to do the technique that we can send out. http://southbaytech.com/appnotes/62%20The%20Small%20Angle%20Cleavage%20Technique%20An%20Update.pdf. http://southbaytech.com/appnotes/61%20SACT%20Prepared%20MBE%20QWIP%20Structure.pdf. http://southbaytech.com/appnotes/59%20EELS%20of%20PLD%20DLC.pdf. http://southbaytech.com/appnotes/55%20GaN Sapphire%20Prepared%20by%20SACT.pdf. http://southbaytech.com/appnotes/60%20PreThinning%20for%20FIB%20TEM%20Sample%20Preparation%20Using%20the%20Small%20Angle%20Cleavage%20Technique.pdf. The first of these files have references for the technique. If you like, have your students give me a call and I will talk to them about the technique. It is a much better approach for these materials than microtoming. For about the cost of a knife (or less), they could buy the equipment and supplies to do it or they could purchase our MicroCleave™ kit. Disclaimer: South Bay Technology manufactures and sells the MicroCleave™ Kit Model 520. Scott D. Walck swalck@southbaytech.com Mon Jun 13

SEM:

cross section study

I am a relative newcomer to the microscopy field and need some advice for the sample preparation for cross section study of fiber material. This kind of film material (polyethylene, polypropylene etc.) has pores structure and orientation. The thickness is around 30–50 microns. It is too tough to break in liquid nitrogen. We had even tried to cut it in liquid nitrogen by a blade. But the pores structure is some distorted. I found cryostat microtomy doesn't work for our case. Is there any trick for this? I got some information from this website before. I still have some questions about it. "(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." Is wax, modeling clay or similar material only used to block end? Is it also used to fill in the big gap between straw and film materials? "(2) Using a syringe, force water into the straw and block the end as above." Is water filled in the big gap between straw and film? "(3) Drop the straw into liquid nitrogen then follow method A part 2 above." Should we cut the sample by blade or break it by tweezers? ***Yun Peng yun.peng@ge.com Tue Jun 7***

I wrote the paper that you discuss so let me try to help you. The basic idea is a media stiffens the fiber and forces it to fracture. Firstly to answer your questions: (1) The wax is simply to block one end. (2) Water may be used to provide a solid interface that will fracture cleanly. (3) Use strong tweezers or grippers (in the UK we would say pliers) to bend the straw unit until fracture occurs. Or (A) Infiltrate the fibers within the drinking with straw a water soluble carbon solution. (B) Once fully infiltrated block one end with wax as above; allow to dry fully (hours). (C) Place in liquid nitrogen until it stops bubbling. (D) Remove and with two strong grippers (UK we would say pliers) bend the straw unit to fracture. We have many times conducted investigation of fiber internal structure by using this method. Steve Chapman protrain@emcourses.com Tue Jun 7

I don't have any comments about the methods you list below, but I would be inclined to try a typical metallurgical cross-section. Mount the sample in a material with similar hardness (epoxy may work fine), and grind and polish to achieve your cross-section. There should be plenty of facilities and people at GE-GRC that are familiar with metallurgical sectioning. Diane Ciaburri diane.ciaburri@gd-ais.com Tue Jun 7

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