NetNotes

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Selected postings from the Microscopy Listserver from November 1, 2010 to December 31, 2010. 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:
cyanobacteria

I have made several attempts to fix and embed for TEM small pellets of unicellular cyanobacteria. The pellets (in Eppendorf tubes) are E.M.-tissue-sized, no more than 0.5 mm on the narrow side. I fix using 2.0% formaldehyde/2.5% glutaraldehyde in 0.1 M cacodylate buffer for 4 hours in the refrigerator, do a brief (10 minute) buffer rinse, fix 90 minutes in the dark in 1% OsO_4 in the same buffer, do another buffer rinse, then do an ethanol dehydration, run through propylene oxide as a transitional solvent, and embed in Epon 812—all standard for tissue and bacteria for me for the past 28 years. But the fixation is horrendously bad, the sections have holes in them that I could work around otherwise, and the Epon appears dark (maybe too long in the osmium for this kind of sample?). The first couple of times I tried this, the glutaraldehyde came from the vendor at the wrong concentration (I sent them the lot no. and they told me I shouldn't have been sent that formulation). But with this last prep I used what should have been good glutaraldehyde (25% E.M. grade from ampoules, diluted to 2.5%). And it looks just as bad. SEM samples (CHO cells) that I used the same good glutaraldehyde on looked fine. If you have any tips or tricks I would very much appreciate your help. ***Scott Robinson sjrobin@illinois.edu Fri Nov 5***

There are a few things that I can think of that might help. For the fixation, you might try fixing in suspension (not pelleted) with higher concentration of glut (e.g. 6%) for longer (overnight at 4°C). Some cyanobacteria make polysaccharide coatings that are difficult for fixatives and dehydrants to penetrate. In a worst case, you might have to re-suspend and re-spin at each step to get decent fixation, dehydration, and infiltration. As for the dark resin, maybe moving to a fresh tube after osmication might help, since osmium can react with plastics. Alternatively, maybe do the osmication in a glass scintillation vial or test tube, then move back to the Eppendorf for pelleting and dehydration, etc. Andy Bowling ajbowling@dow.com Sun Nov 7

For several years, we had research students preparing isolated cyanobacteria or lichens, which contained cyanobacteria. The walls of the vegetative cells could be a problem, often the heterocysts were even worse and some storage bodies had a habit of dropping out (e.g. polyphosphate). Sometimes the only good results were obtained with $KMnO_4$ fixation, prolonged dehydration and embedding for extended periods in low viscosity resins such as Spurr's. Our researchers got good results in the end but it did seem like a bit of a black art. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Mon Nov 8

We work with budding yeast for TEM analysis all the time. Yeast also has a thick cell wall, in a sense, similar to cyanobacteria. The OsO_4 cannot penetrate through the cell wall and you have to use enzyme to remove the cell wall before applying the OsO_4. We use Zymolyase 100T for yeast cell wall. As others suggested, potassium permanganate can be used as an alternative to OsO_4. Here is a reference. Let me know if you need a PDF copy of the paper. H Yang, Q Ren, and Z Zhang, "Chromosome or chromatin condensation leads to meiosis or apoptosis in stationary yeast (Saccharomyces cerevisiae) cells," *FEMS Yeast Res* 6(8) (2006) 1254–63. Zhaojie Zhang zzhang@uwyo.edu Mon Nov 8

Specimen Preparation:
difficult infiltration

Does anyone have specific advice for what to do with difficult-to-infiltrate single cells? I'm looking for recommendations on resins and on time schedules for infiltration and polymerisation. I'm working with unicellular eukaryotic algae (2–5 microns, cell wall about 100 nm thick) and myxozoan spores (10 microns, spore wall about 500 nm thick). I do not have positive evidence for what the walls are made of, but it's not obviously mineralized. Both are very susceptible to poor infiltration and extracted-looking cytoplasm. The literature is uninformative about how long the good published examples have been infiltrated. Does "the longer, the better" apply here? Or, will that make the extraction worse? Or, wouldn't it matter on the timescale of a week in resin? Unfortunately, with the EM unit closing for Christmas, and the fact the myxozoan spores come from an endangered species, I do not have the option of trying different lengths of time to see what is best. I guess if people were adamant that longer is definitely better, I could leave them infiltrating until the unit opens again on the 5th January. ***Giselle Walker gw265@hermes.cam.ac.uk Tue Dec 7***

I find myself in a similar situation to yours. I'm a mammalian biologist that suddenly needs to process and embed algae for someone, and am having trouble with fixation and embedding. I also am not finding a good protocol. If someone sends you a good protocol, would you mind sharing it with me? I would appreciate it! I have a sample that I'm currently giving a try at. I started fixation with 4% paraformaldehyde/2.5% glutaraldehyde at 37°C, and cooled the sample from there in the refrigerator. I left the sample fix over the weekend, then buffer rinsed overnight, and osmicated for 2 hours at room temp in 1% osmium. I rinsed for another hour in buffer and then started an acetone dehydration, 15 minutes each in 35%, 50%, 70%, and 95% acetone, 4 changes of 100% at 15 minutes each, then 2 changes of 100% propylene oxide. I spent hours gradually going through propylene oxide:resin infiltration, thinking that I would get good infiltration, but the algae floated in a 3:1 mix of resin:propylene oxide so I switched to acetone and resin, thinking that maybe I hadn't fully dehydrated the algae. I left the algae overnight in 50:50 acetone:resin at room temp, then went to 3:1 resin:acetone the next day and the algae still floated. I just continued on to higher resin concentration. I went to 100% resin for a day, then overnight in the refrigerator in resin. Now, I'm going a second day in resin on a rotator, then into the 70-degree oven at the end of the day. I don't know what else to try. I don't have access to an E.M. grade microwave, although I've heard that it is the best way to handle these tough samples. If you hear of a better way, would you send it along? Edward Haller ehaller@health.usf.edu Thu Dec 9

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As I mentioned to Giselle, I am familiar with Monica Schoenwaelder's work on brown algal zygotes, and her search for a protocol that would preserve phenolic vesicles—physodes, intact for TEM. Here's an extract from one of Monica's papers note the slow infiltration: http://onlinelibrary.wiley.com/doi/10.1055/s-2000-9178/abstract. For electron microscopy, eggs, zygotes, and embryos were fixed in a glutaraldehyde/paraformaldehyde fixative (Schoenwaelder and Clayton, 1998a[37], b[38]) for 23 h. They were then rinsed three times in sodium cacodylate buffer (2% sodium chloride and 0.1% calcium chloride in 0.1 M sodium cacodylate buffer), and post-fixed in 1% osmium tetroxide in buffer for 2 h. Specimens were rinsed three times in distilled water to eliminate excess osmium tetroxide and then dehydrated through a graded acetone series of 10% increments, before three changes in 100% acetone. The specimens were infiltrated very gradually (over 2 weeks) with Spurr's resin (Spurr, 1969[42]) (medium/hard mixture) before polymerization at 60°C in plastic BEEM capsules. Monica also mentioned that she started her infiltrations with 1% resin increments, perhaps increasing from 1% to 5% over day one, then 5% to 10% day two, then increased in larger steps in the middle range of concentrations, and then slowed down again after reaching 90% resin. (This is what I remember, you could check her earlier papers for details—our library doesn't get *Phycologia* any more.) The brown algal zygotes are not only quite dense but also have surprisingly impermeable walls—the wall pores allow rather slow resin monomer permeation, but the small solvent molecules can escape quickly, of course, and if the gradient across the wall is too great, the zygotes crumple inwards. A further trick, outlined by Geoff Wasteneys and colleagues, is to freeze the cells briefly, then thaw—to make a few cracks in the cell wall. See Wasteneys et al. 1997. Freeze-shattering: a simple and effective method for permeabilizing higher plant cell walls. *J. Microsc.* 188:5161. Essentially, you freeze the tissue between two slides in liquid nitrogen then press down gently while frozen, then thaw. This works pretty well, but requires a bit of practice. Rosemary White rosemary.white@csiro.au Thu Dec 9

Specimen Preparation:

osmium safety

I'm gearing up to teach introductory electron microscopy to undergraduate biology majors again this Spring and have hit a few safety road blocks on which I'd greatly appreciate your advice. We don't have a dedicated health and safety unit at my University. Therefore, the first thing that I need is a full-face respirator in the event that someone drops osmium because it's all up to me to clean it up. My first question is, do I need filtered air or supplied air? Any other suggestions? Secondly, I have a faint memory of there being a way to reduce the toxicity of osmium waste by binding the osmium to oil. We are trying to reduce the toxicity and save the University some cash in waste disposal fees because it will be cheaper to deal with less toxic waste. Now, a couple of other question related to the class. I know that I'm going to run into some trouble when I tell our budget keepers that I've got to order new supplies because 2 year old glut is not acceptable. We have a heavy focus on field science, so the expense of this class comes as a shock. Does anyone have any insight as to the "shelf life" of unopened ampoules of glutaraldehyde? Maybe this would be a better buy this year. Lastly, as a plant person, I'm struggling with how to get my students experience with animal tissue because I lost my colleague who used to sacrifice rats for his research and lend me some tissue for my class. I do have a connection to a local vet and am wondering if anyone has any advice as to whether having him pop some of the tissues that he harvests from his patients into vials of Karnovsky's would be a good option. My students may get a lot of cat testicles and ovaries, but at least they'd get some animal tissue! ***Kristen Lennon kamlennon@yahoo.com Tue Nov 9***

First thing is don't panic. Not all undergrads are disaster in the making. Lots of graduate students are the same. I have engineering students who have never used a pipette or pH meter, let alone worked in or near a fume hood! I've also had undergraduates who have been fantastic students in all respects. A couple of mandatory rules will help, including handling all chemicals in hoods at all times! This should limit the inevitable small spills to at least fairly safe confines. I also make students work on enamel trays in the hood to further confine spills. And of course all students need to wear gloves when working with chemicals of any kind. Regarding osmium . . . fill a 1 liter polypropylene bottle (preferably wide-mouth) about 1/3 full with vegetable oil . . . the stuff you use for cooking. Add osmium waste. The osmium will react with the oil, which renders it much less reactive. We always treat osmium waste this way to aid safer disposal. Don't worry about the glutaraldehyde. As long as it has been sealed in vials it should be fine. Remember this is a basic class and you are not trying to optimize sample preparation. Only problem I have had with older glutaraldehyde is when the concentration is above 25%. In this case the glutaraldehyde was cloudy when diluted. However, we do try to use up glutaraldehyde within a year just to avoid potential problems. We also try to use it within a week once the vial is opened and the glutaraldehyde is diluted. As to animal tissue, is anyone at your university working with mice or zebra fish that could provide some tissue? Perhaps someone is working with fertilized eggs and you can get a chicken embryo. Good luck . . . it is always a challenge teaching lab courses but can be very satisfying as well if you actually turn on some of the students to science research . . . especially microscopy. Debby Sherman dsherman@purdue.edu Tue Nov 9

The toxicity of a product is a function of its concentration and the amount that is absorbed, so use diluted solutions and don't let the students play with large solutions and you have made a big step in the security. The osmium solution must never leave the hood, so I don't see why you would need a full-face protection. If a few drops have left the hood, safety (closed) goggles should be enough. Otherwise Debby gave good advice. To neutralize osmium one needs unsaturated fatty acids, generally sunflower oil is cheap enough to be used. As for the samples your idea is good but the vet should better slice the organs or alternatively the organs may be conserved in ice (for 1–2 hours should not be too bad). Then you can slice them and fix them yourself (or the student can do it). Skin should also not be too hard to get from a vet and the histology is interesting and not too complicated. Stephane Nizets nizets2@yahoo.com Wed Nov 10

First, to echo other responders, don't worry about teaching to undergraduates. We have a microscopy major within our Biology B.Sc. program and routinely teach TEM and SEM to undergrads. I've never found that undergraduates are a big safety problem. (Engineering grad students, now . . .) Teach proper techniques (not over-done panicky Safety Committee Ohmygodosmium!) and they'll be fine. Do emphasize that OsO_4 is highly volatile and must be used in a hood. Show them a blackened OsO_4 storage container and the inside of your fixative refrigerator. They'll get the point. Keep a spill kit handy and show them how to use it. OsO_4 waste: as others have written, use vegetable oil in a plastic container (not glass—glass breaks, then you have a nasty mess), but find the cheap highly polyunsaturated oil, like Canola. The more unsaturated the oil, the more it binds up OsO_4. Also, add kitty litter to the waste container. This keeps the oil from sloshing around and increases the surface area of oil available to bind OsO_4. Have a separate wide-mouth container for solid OsO_4 contaminated waste—transfer pipettes, gloves, etc. Emergency respirator—filtered

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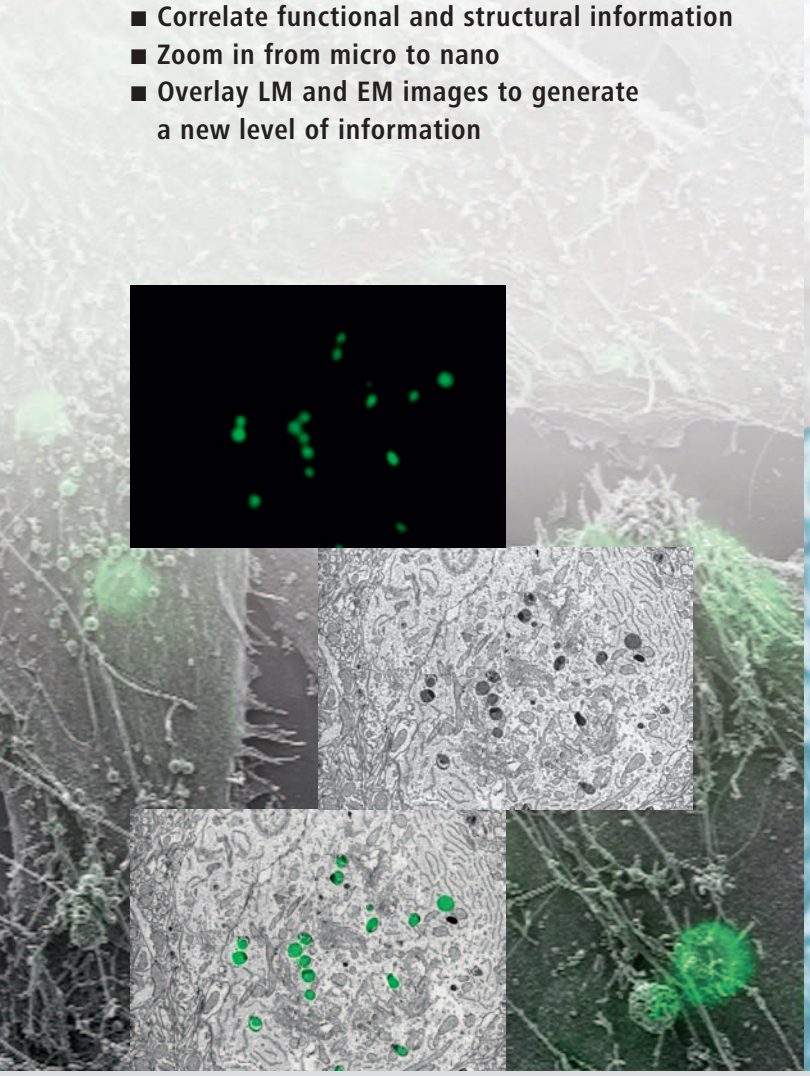

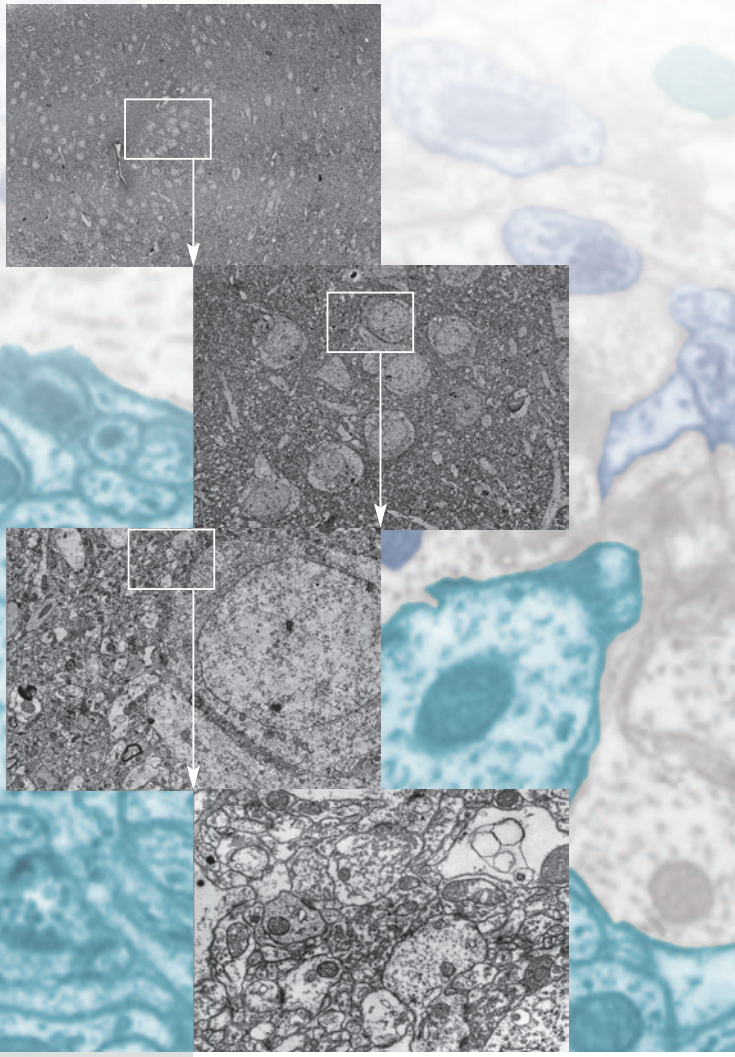

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is fine if it's the right filter. But mostly, have kitty litter and oil handy to dump on any spill. But, if the OsO_4 is kept in the hood, then any spill should be in the hood—preferably in a tray—so a respirator really isn't necessary. Re: samples. You can get away with using tissues from a local vet or other people's research, unless you have an active animal-care committee and animal research protocols. Then you'll likely discover that you must have your own animal tissue protocols even if you are using samples taken from animals used in research or brought in from off campus. But if you use insects like crickets—used for reptile food—or the cockroaches and spiders hanging around your building, then you have no issues with animal care committees, animal use protocols (there are laws and regulations involved here beyond your campus regulations) and so on. (Or isopods—pillbugs and sowbugs—or worms or etc.) Fruitflies. Plus, you get really neat specimens: contracted/relaxed muscles, Malpighian tubules, venom and silk glands, and lots more. And there are lots of EM images in the literature and the 15-volume *Microscopic Anatomy of Invertebrates* published by Wiley-Liss and Miriam Rothschild's beautiful "Insect Tissues via the Flea". Or, brine shrimp (Artemia) are easy to culture. All the specimens for this would be free. Note: just dumping testicles and ovaries into a jar of Karnovsky's would be a good exercise in poor preservation and why one minces tissue for EM fixation. Phil Oshel oshel1pe@cmich.edu Wed Nov 10

I would like to broaden the topic a little and request advice. Osmium. I have been hording unreacted waste in Kilner/Mason jars in my fridge. I suppose that when it is reacted with oil then it is no longer volatile. Is this mixture in a polypropylene bottle with an ordinary screw lid a satisfactory container to pass on to a waste disposal operative? Supplementary question: Phil—Kitty litter—I presume you mean the mineral type—how much do you use? e.g., 10% of the bottle's volume? Waste glutaraldehyde in buffer. This will probably still be volatile. What is a satisfactory container to pass on to a waste disposal operative? Dave Patton david.patton@uwe.ac.uk Wed Nov 10

No. The osmium is volatile enough to escape a tightly capped jar. The inside of your refrigerator is pretty black from osmium, I imagine. Parafilm (or what you have in the UK) the ever-loving cap of the jars. Kitty litter: Yes, the clay type. This is less important than is using something absorbent that the oil will cling to. This is what increases the effective surface area of the oil, making more of it available to the osmium. I eyeball a layer a couple of centimeters deep in a US gallon/4 L jug, then add around 200–500 mL or so of oil. As waste osmium accumulates, I'll add oil and maybe litter if it looks needed. Waste glurarаldehyde: A tightly capped plastic bottle. Glutaraldehdye is less volatile than osmium, but Parafilming the cap isn't a bad idea. Mind, though, this can also depend on your local laws and regulations, and the local hazardous waste disposal people. They may have other ideas, in which case you must do things their way. Note that accumulating hazardous waste can be a particular problem. Here in the US, there are laws about how much waste of what kind can be accumulated in a local area—like an individual lab—and in a waste collection site and so on up the ladder to the waste disposal/processing area. I'd think the UK has similar laws. And in what the waste is accumulated. Your refrigerator hoard of unreacted waste may be a serious problem in this regard. Phil Oshel oshel1pe@cmich.edu Wed Nov 10

First, let me strongly suggest you not store unreacted waste in Mason jars in your refrigerator. My experience is no single jar seal is sufficient to contain osmium vapor. I store my 2% osmium and osmium waste in my fume hood. My 2% osmium stock is in a tightly sealed orange-cap Schott bottle. The orange cap has gone completely black. The entire Schott bottle is kept inside a clear plastic polycarbonate jar. The polycarbonate has gone completely black. I have seen, in other labs in the past, refrigerators with blackened walls due to storage of osmium as you suggest you do. As a general rule, store your osmium in double containers and keep them in a fume hood. Secondly, it is not allowed at my University for an investigator to "process" hazardous waste without a written protocol that has been approved. I can't simply put my osmium waste in oil such as Debbie suggests. I think I once asked about that and was told not to do that. I don't know the rationale behind this denial. Tom Phillips phillipst@missouri.edu Wed Nov 10

Wow. Really? I've put osmium waste in oil everywhere I've been, or seen that where I've gone. I've surprised to see putting osmium in oil as "processing" and not "storage" (or some similar synonym). Mind, the simplest is just to do it, and state its part of the experimental protocol. The osmium-in-oil (aka osmium dioxide bound to unsaturated carbon-carbon bonds in an oily polymer) is the waste. Not the osmium removed from the specimens. Phil Oshel oshel1pe@cmich.edu Wed Nov 10

Years ago, a guy in charge of our hazard waste told me one rationale for not allowing any "processing." Corporations had to pay $XXX dollars to get rid of barrels of anything that contained what was classified as "hazardous." But barrels of very low ^3H radioactivity were allowed to be poured down the drain with plenty of rinsing under federal guidelines. The scam that some industrial corporations were doing was adding a small amount of radioactivity to their waste stream. Those "hazardous" barrels were now under federal "radioisotope" waste restrictions and therefore could be poured down the drain at a significant cost savings. I believe this loophole has been plugged. We also can't call anything in our lab waste nor can we label it as "waste." We only have "Used, unwanted chemicals" since "waste" implies it has to be handled with a special protocol and the "used, unwanted chemicals" folks don't want their hands tied. The EHS folks at my university have some tedious rules but one thing they do deserves great credit and should be copied at all universities. They do a special pickup for any "unwanted chemicals" which includes any chemical that you use 1 mg in an experiment once or twice and don't need the remaining 199.9 gm in the original bottle. These chemicals are stored in their facility, listed on a website and available for any registered user on campus to go and get for free. Lots of chemicals don't go bad just because they are a year or ten old! In many ways this is no different than using an older bottle on your own shelf or borrowing from a neighboring lab. I can't tell you how often this has saved me money and allowed me to do a pilot experiment I wouldn't have tried if it meant ordering some $100 bottle. If your university doesn't have this, you should agitate for it. It saves money and reduces the toxic waste stream. Tom Phillips phillipst@missouri.edu Wed Nov 10

Here we must label things as hazardous waste, then the chemical stockroom people pick it up and haul it away. I do like the unused chemicals idea. UW-Madison does that, and you wouldn't believe the grams of chloroauric acid I got free that way—and other such metals. Making colloidal gold, etc., for labeling was really cheap. CMU doesn't do this. But—glad you reminded me. I think I'll suggest this. Phil Oshel oshel1pe@cmich.edu Wed Nov 10

Specimen Preparation:
lead staining

Post-staining with triple lead, I post-stain the immunolabeled samples with triple lead. I try to avoid precipitate formation using NaOH pellets around the staining chamber; however, there is a lot of precipitation on the grids even if I wash them several times in MilliQ water. Is it advisable to wash the grids in 0.1 N NaOH solution and

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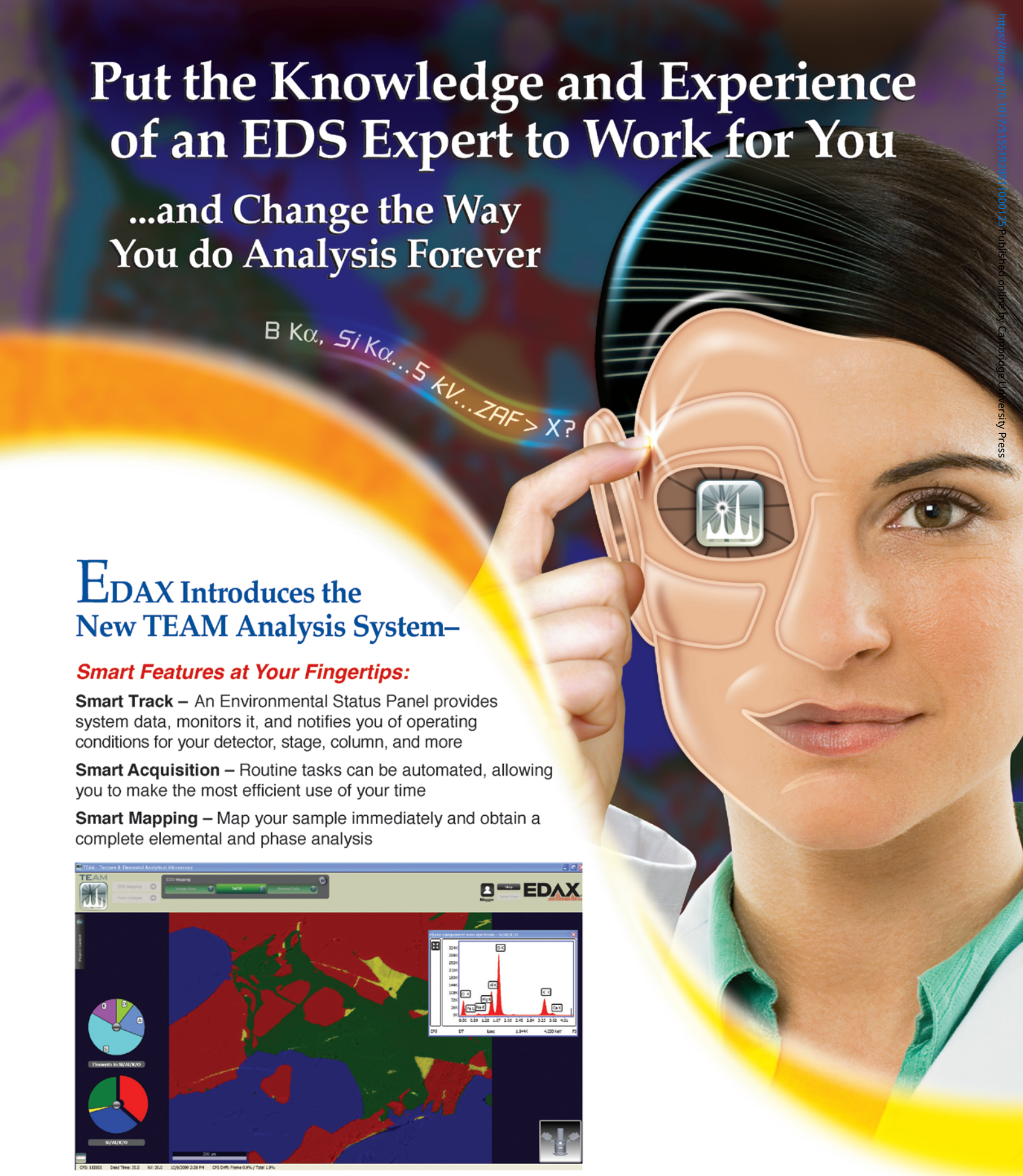

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re-stain them again? If I wash them in NaOH solution will it affect the immunolabeling reaction? ***Chaitali Dekiwadia dcd@unimelb.edu.au Mon Nov 29***

We seldom use lead stain on immunocytocheistry sections but rather use a smaller objective aperture to boost tissue contrast, at least for initial viewing. Whenever there is precipitation it is always good to look at an unstained section to check if the precipitation is in the section rather than on it as a result of the staining procedure. After checking that it is fine, you can stain the same grid for imaging if you wish. Did you syringe-filter the stain before use or at least allow the stain to settle a while before taking the stain from near the top of the tube? I have also found less frequent lead precipitation on sections if I have the grids submerged in non-acid water drops (1 pellet of NaOH in 0.5 L) before putting them into the lead stain drops. I have also used water from this same bottle for a brief rinse after the lead stain followed by some good water washes. I have not tried to wash off precipitation recently for I now pick up more grids than I need usually five. That way if I stain two, I have three in reserve if there is a need to check or correct a staining problem. Many years ago I did attempt to wash off lead precipitation but I must not have done it correctly since it did not work for me and I have not done it since. Hopefully others will add to my comments especially to the point of washing the grids in 0.1N NaOH solution. Patricia Stranen Connelly connellyps@nhlbi.nih.gov Mon Nov 29

My understanding is that to remove lead precipitate from sections, it is necessary to incubate the sections on an acidic solution. This is why lead staining has to follow uranyl acetate staining since the acidic uranyl solution would remove the lead. My guess is some precipitates will be refractory to being re-dissolved and removed but it is worth a try. My recollection is that once you have viewed a grid in the TEM, it is especially difficult, if not impossible, to remove precipitates so this is really only an option if you stained numerous grids and haven't looked at them all. Tom Phillips phillipst@missouri.edu Mon Nov 29

I have tried all different ways to avoid lead precipitation such as preparing the solution in boiled MilliQ water, spinning down the lead citrate solution before using it. However, I was interested in the protocol that suggested washing first in 0.01N NaOH after lead staining and then successive washes in boiled MilliQ water. My question is won't the NaOH wash precipitate the uranyl acetate stain? As I read few protocols that stated the uranyl acetate gets precipitated in the presence of NaOH? Chaitali Dekiwadia dcd@unimelb.edu.au Wed Dec 1

A 0.1 NaOH may be helpful but since it can form $NaHCO_3$ by combining with atmospheric CO_2 it may exacerbate the problem if you aren't careful since this can lead to $PbCO_3$ precipitates forming. But it won't impact the uranyl acetate since the original lead solution was equally basic and that doesn't cause problems. I don't believe most people find a NaOH wash necessary so it is probably better to prevent the problem rather than trying to reverse it if possible. Like much of what we do in science, half the steps are unnecessary but we just don't know which half. A NaOH wash might be essential if you have a different less desirable step upstream from this one but unnecessary if you have a working, streamlined approach. Be careful that you aren't breathing on your grids while staining them since the CO_2 in your breath causes precipitates. I have caught many students intently staring at the grids from a short distance while trying to pick them up off a lead citrate drop. Everyone who has ever done a lot of TEM has had this staining problem. There are dozens of papers on how to prevent it which tells you that there is no one method that works for all or we wouldn't need to keep coming up with the solution. Tom Phillips phillipst@missouri.edu Wed Dec 1

Specimen Preparation:
chloral hydrate alternatives

I'm finding that chloral hydrate is increasingly difficult to purchase even for a school department, being a Schedule IV Drug. This is unfortunate, as it was once a basic compound for use in microscopy, for solutions such as Hoyer's mounting media, Melzer's reagent, or on its own in an aqueous solution. Does anybody have suggestions for other compounds that might serve as a good clearing agent, without depending on being a strong acid or base for their clearing activity? ***Peter Werner germpore@sonic.net Thu Dec 2***

We use methyl salicylate. Around here, mostly for embryos and shrimp. Works well for fluorescence microscopy, too. It does require dehydration with ethanol. Philip Oshel oshel1pe@cmich.edu Thu Dec 2

So I inquired the other day about chloral hydrate substitutes, and one of the compounds that sounds promising is 2,2-thiodiethanol (aka TED or thiodiglycol). (A big thanks to Keith Duncan for the tip, BTW.) Article here: http://onlinelibrary.wiley.com/doi/10.1002/jemt.20396/pdf. Does anybody know whether this compound is reactive with iodine or potassium iodide if they were mixed in the same aqueous solution? I'm not a chemist, so I wouldn't know offhand from looking at the classes of compound whether the two are reactive. My concern is that they are miscible without reacting and either creating another compound or creating a cloudy precipitate. Or worse, creating a compound that's highly toxic. I will note that TDE is a precursor of mustard gas, which is why I'm particularly concerned that it not be reactive with a halogen like iodine. Has anybody used TDE with iodine stains? Peter Werner germpore@sonic.net Thu Dec 2

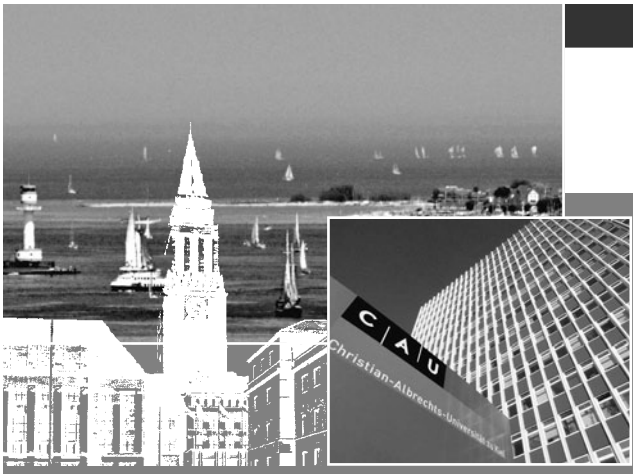
As also not a chemist, I cannot translate between the names and the chemical structure of TDE; however, if it is the sulfur equivalent of ethylene glycol—that is, with two SH groups on it—it should be a powerful reducing agent, which would react strongly with an oxidizing agent like iodine. Bill Tivol william.f.tivol@aero.org Thu Dec 2

I don't have an answer to your specific question, but I have extensive experience using a variety of iodine reagents for working with fungi and lichens (see: Common 1991 Mycotaxon 42: 35–41). Different iodine reagents give different results in many cases. The reactions are very sensitive to the concentration of iodine and the other components of the reagent. I discuss a variety of reagents in the paper. For most of my purposes Melzer's reagent was not the most useful, and it is certainly not the best for detecting many reactive materials. In any case I doubt you could find another combination of components that would give precisely the same results as Melzer's. Ralph Common rcommon@msu.edu Thu Dec 2

Specimen Preparation:
differences in glass for microscope slides

I was wondering if anybody can explain what qualitative difference exists, if any, between different brands of plain glass slides. For example, over on the Ted Pella page (http://www.tedpella.com/histo_html/slides.htm), for a 2-box pack of 144 slides, the price varies from about $10 for the Pella Economy Slides, all the way up to $40 or $60+ for Gold Seal or Corning slides. My question is, is there any advantage or optical difference with the more expensive slides? Corning advertises theirs as "water white" glass, which I imagine implies something about the transmittance or refractive index, but I'm not sure. ***Peter Werner germpore@sonic.net Sun Dec 12***

Water-white glass is low iron and has 98-99% transmission. If you ever have looked at a slab of window glass on edge, you will have



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notice the edge looks green since the glass absorbs some red and blue light. I guess I have seen that same phenomenon when I have looked at a new box of microscope slides where they are packed together and the edges look green. I intend to compare my different brands of slides when I go into work later today to confirm that recollection. I wonder how important the effect is in microscopy for brightfield work. These seem characteristics that would be important for the coverslip glass but less so for the slide to me since the amount of incoming light that passes through glass is usually not limiting. The color balance of the light going through the glass is partly dependent on the power to the tungsten bulb which is why microscopes used to always have a button for fixing the power of the bulb when taking color slide film in the old days and one needed to take into account whether you were using daylight or indoor style film. Most digital cameras use white balance to eliminate this problem so I don't know if the glass absorption of some wavelengths is a significant issue for regular bright field work. On the coverslip side, getting all the light from fluorescent specimens would be beneficial but in this case both the excitation and emission light are passing through the coverslip. Tom Phillips phillipst@missouri.edu Sun Dec 12

I only use Corning 2947 LM slides. The big problem is finding good cover slips that are not made in China. These units come pre-cracked. Wonderful. I pay more for quartz slips. Whatever the color transmittance is, I can correct it with digital capture software light balance. Gary Gaugler gary@gaugler.com Sun Dec 12

Quartz? You're worried about the variations in coverslips (Cracking? thicknesses?) and so you use Quartz? Hmm, what about the index of refraction? Okay, fused quartz index of refraction is 1.4585 (1.47 to 1.45), which is really close to 1.473 glycerol (general mounting medium particularly with the anti-fade agents). And it is closer to water at 1.33 for wet mounts versus glass at 1.515. Actually quartz does sounds pretty good. Perhaps I'll give it a try, but now I have to buy some 1.4585 oil. As for the slides, I would have to agree that for Brightfield work I have used both the green and the white slides with little difference. I generally avoid the ultra cheap really green slides, and go for the cleanest—none are really clean but it makes it easier to clean them. And in general most folks do not even think about lamp color temperature or accurately setting white balance in the digital cameras. For reflected light, epi-fluoresence or confocal work, the slide color does not matter. Richard E. Edelmann edelmare@muohio.edu Wed Dec 15

Specimen Preparation:

electrothinning

I have been attempting to electro-thin 3 mm discs of CMSX4 for TEM examination but am having some trouble obtaining useful samples. I am using a Struers Tenupol-5 with an etchant of 5% perchloric acid in methanol. I have tried many voltages, sample thicknesses and electrolyte temperatures and from the 50 or so discs I have tried, only 1 is of any use! I have a feeling I may be using the wrong electrolyte for this application. Does anybody have any experience of thinning this material? If so, could you please advise me which electrolyte to use and perhaps suggest some starting conditions? Any help that anyone can give will be very much appreciated. ***Mark Jepson m.a.e.jepson@lboro.ac.uk Wed Nov 24***

I use 4% perchloric in methanol at –40°C in a Tenupol. It is my universal polishing solution and I have had great results with everything from mild steel, austenitics, superalloys, rare earths, titanium etc. The trick with electropolishing is to forget about voltage and concentrate on current. It is current that controls the electrochemical dissolution; the voltage will vary all over the place as the conductivity of the solution changes with increasing use (more metal salts). I always discard my solution after use. Metal perchlorate salts, which crystallize out on bottle stoppers, can be explosive. For double-side thinning I use a current of between 120–200 mA. Start at the low end and increase the current in 20 mA increments for subsequent specimens. Examine them to see where the sweet spot is. If you are right on the edge of good electropolishing conditions, you might find what works one day, doesn't the next. If your current is too low, you will find your specimens to be badly oxidized. If it is too high, your specimens will perforate very quickly (<20 s), and you have pinholes that look like apertures. This is based on specimens ground to around 80ums thick. If you are working with ferromagnetics, you might want to grind a little thinner, to reduce the effect on the TEM fields. Electropolishing times will typically be between 20 and 60 s. Some other things to think about: Flow rate is not too critical; I use 3 on a scale of 1 (slow) to 10 (fast). Temperature is very important. Low temperatures reduce oxidation and increase viscosity, favoring the establishment of good electopolishing conditions. You didn't mention if you were cooling. If not it is a must with this solution. A small Dewar, some silicone tubing and a diaphragm pump to draw the LN_2 through the Tenupol's cooling loop and you're in business. Cleanliness is paramount. Clean all equipment with methanol before and after use. Make sure there is no water contamination in any of your solutions/equipment. Set the optical sensitivity to maximum, and get the specimen out of the Tenupol and into a beaker of methanol immediately the perforation alarms sounds. When electropolishing stops, corrosion starts and you might get etch pitting. Wash your specimens very, very thoroughly with pure methanol from a wash bottle and air dry—do not use acetone. Store specimens in a desiccator, never under vacuum. I place all perchloric acid contaminated tissue waste into a very large beaker of water as I go. At the end I rinse this through with fresh water, then dispose of it sealed inside a Ziploc bag, so there's no danger of spontaneous combustion. Dave Mitchell drg.mitchell@sydney.edu.au Wed Nov 24

Microtomy:

thick sections

Some of my students have asked me to inquire about a problem they are having with thick plastic sections falling off their glass slides. Any advice or tricks of the trade? These are 1 µm or so plastic sections, dried down in a drop of water onto a clean glass slide. The sections seem to slip off the slide during rinsing after toluidine blue staining. These are very good students who will appreciate any replies. ***Jon Krupp jkrupp@deltacollege.edu Wed Nov 10***

What temperature are they using to dry down the slides & for staining? We use around 90°C and gentle washing. Phil Oshel oshel1pe@cmich.edu Wed Nov 10

Are you using adhesive slides? If yes, try to leave the slides with sections 20 min on the hotplate at around 58°C, before staining. Joe Siegmund jsiegmund@7thwavelabs.com Wed Nov 10

I hope your students will be delighted to know that when using Epon or Epon-substitutes (like glycidether 100, Embed or also LADD LX-112 epoxy resins), I did not have problems staining large (up to 4 × 5 mm) 1 µm sections with a multi-step polychromatic staining sequence (at least one step 8 min @ 80°C) if the sections were left on a hotplate @100–120°C for at least 5 min. Certainly there could be differences in treating (heating) sections on a glass slides for other resin types, critical in my opinion too perhaps is age and cleanliness of slides. Before use, mine are batch-treated in diluted HCl-ethanol solution, rinsed thoroughly in distilled water and dried in a dust-free location. Heating sections to such high temperatures before staining in my opinion will not have much adverse effect in terms of staining quality or intensity. Wolfgang Muss w.muss@salk.at Wed Nov 10

My hot plate is rather hot but I have not taken the temperature and it is off. The most frequent reason for my sections falling off the slide is impatience. When I think it is time to put the stain on I stop and wait another half minute. Total time would be maybe 3 minutes for I use a rather small drop of water most times. I guess at least a minute or so after the water has evaporated would be fine. I would go nuts if I waited 20 minutes. For the staining I wait until a small ring can just be seen at the stain drop boundary, rinse immediately and dry down again. The time for this step can be shortened considerably by absorbing most of the water on the slide around the section with any type of tissue or paper towel. If a section is much thicker, say 2 microns it will, in my hands, still wash off if not treated very gently. I watch for a floating section, rescue it with a loop, pass it through several drops of water to clean off the stain and then dry it again onto the slide. The suggestion of using treated slides is a good one. Patricia Stranen Connelly connellyps@nhlbi.nih.gov Wed Nov 10

Immunocytochemistry:

immunogold labeling bacteria

Attempting pre-embedding immunogold of bacterial suspensions. First round reveals complete absence of 12 nm gold particles which worked for post-embedding immuno-electron microscopy. Did we spin off the particles (3000 rpm for 5 minutes) during pelleting? Does anyone have an ideal protocol? ***Walter Bobrowski walter.bobrowski@pfizer.com Nov 5***

You wouldn't spin them off. I presume you are aiming for a surface antigen. Steric hindrance might block access to the antibody in pre-embedding. You could try a nanogold secondary and then use a gold enhancement step to maximize signal. Does a fluorescent-tagged secondary work at the LM level with this approach? Tom Phillips phillipst@missouri.edu Fri Nov 5

Yes, if this is about labeling a surface antigen it could very well be steric hindrance. In E coli LPS chains can be responsible. To reach antigens in the outer membrane we needed to use cryo ultramicrotomy, intact cell labeling did not work. This was described in WF Voorhout, JJ Leunissen-Bijvelt, JLM Leunissen, and AJ Verkleij, "Steric Hindrance in Immunolabeling" *J Microsc* 141(3) (1986) 303–10. Jan Leunissen leunissen@aurion.nl Fri Nov 5

Image Processing:

stitching images

Can anyone suggest good software to stitch multiple SEM images together to make one large image from multiple frames? The software I have tried all assumes they are landscape images, and accounts for angle changes, or a curved lens, and I end up with a curved image. ***Sharon Lackie sharonl@uwindsor.ca Fri Dec 17***

Type "autostitch" into Google and you will get the program developed at UBC that will do just that. I don't think it will distort the images and it is free for Windows. Mary Fletcher maryflet@interchange.ubc.ca Fri Dec 17

Thank you, Mary, but we do sometimes see distortion in our stitched images when we use "autostitch"—not always, but enough times to cause us problems. Our images are from petrographic thin sections, i.e. transmitted light microscopy, if that might make a difference. Are we alone in seeing this? If so, we must consider that it is our usage, not the software itself, so I would appreciate replies either way. Thank you, Barrie Wells

Depending on the manufacturer of the SEM, the software might be available from them, double check with applications support. I have used a commercial software (not free) called SIGMAGIS from SmartImaging Technology. The company can assist you with writing scripts or automating the process if you have large data files. My image arrays range from 100 × 100 to much larger. The software will compensate for lens distortions etc. For small jobs, you can investigate either Google or Microsoft products for Gigapixel imaging downloadable from the web, also ImageJ and Digital Micrograph has scripts that can stitch few images together.

Photoshop allows one to select whether stitched images are corrected for perspective or cylindrical layouts or just be repositioned without correction. It is the Photomerge function under Automate. Tom Phillips PhillipsT@missouri.edu Sun Dec 19

I have had the same problem, as most "panorama" software are built to be used with images coming from a camera, they suppose one is in a conical projection system. There are a few plug-ins for ImageJ (MosaicJ, Large montage, Patchwork), which are more or less manual. I tested only "Large Montage." I got some good results with Hugin, an open software using the Panotools library. (I use it on Linux, but it exist too for Win and OSX). As it calculates the focal length, it jams on the results it gets, as the value is much too low for what is normally wait! Focal length of 0.5 to 3 mm and a field of view from 100 µm to a few mm are not common values for panorama makers! One must force it to accept these values, and it works further. With SEM images, I had always to set the control points manually, as the B&W makes the automatic location process a bit more complicated. It depends of the type of picture you have. The projection type is set to rectilinear. One can either give an initial field-of-view value (or a focal length) or let it calculate them itself, and fine-tune the result. In that case, in the menu "Optimization," one chooses the option "all." At the end of the computation, one can again modify more or less the focal length, to minimize the distortions of the montage. I had nice results with sets of up to 20–30 images; I didn't try with more images. Jacques Faerber jacques.faerber@ipcms.u-strasbg.fr Mon Dec 20

Media Cybernetics, Image Pro Plus software can stitch images together. This software is written for images from many different sources, whether they are taken from an optical microscope, electron microscope, X-rays, or ultrasound, etc. I have never seen distortion at the edges when stitching the image back together. You need to have some overlap between each image, so the software can see where to line up landmarks though. We use this software all the time. Cheryl Rehfeld csr@meyerinst.com Mon Dec 20

You could try Zeiss or Raith and get their SmartStitch app. It is dedicated to SEM stitching. There are other apps that do stitching but this one is quite good. Gary Gaubler gary@gaugler.com Tue Dec 21

Instrumentation:

cleaning camera sensor

I've been reading up on digital camera maintenance, and most of the sites/literature on the topic concerns digital SLRs. I've been looking at this site in particular: http://www.cleaningdigitalcameras.com/ I was wondering, first, if microscope cameras, such as the Zeiss Axiocam, Photometrics CoolSnap, or QImaging QICAM, have an anti-aliasing (aka "low-pass") filter in front of the actual sensor the way that digital SLRs do. If so, do microscope camera occasionally require the same "sensor cleaning" that digital SLRs do? The only time I've looked straight inside of a microscope camera was when changing the mount on a Zeiss Axiocam, and in that case, it seems like the sensor was very exposed, with no glass between the front of the camera and the sensor. If this typical of microscope cameras, what should one do if dust gets on the sensor? Obviously, cleaning the actual sensor is a great deal more risky proposition (if it is doable at all) than typical "sensor cleaning" that actually means cleaning the anti-aliasing filter. Obviously, microscope cameras generally stay mounted in a lens-down position and without regular changing of lenses, and so have the inside of the camera much less exposed to dust and dirt exposure than would

be typical of an SLR, hence the need for cleaning should be much less frequent, in any event. Also, if there's a website or publication that gives general methods for care and maintenance of microscope cameras, please let me know. ***Peter Werner germpore@sonic.net Wed Dec 22***

One of the scopes I worked on had a CCD mounted just below the film camera, which the user routinely collected data with. When the resolution of the CCD started to deteriorate, it was discovered that there was contamination on the sensor, so the service person gently washed the sensor with ethanol. This restored most of the resolution to the CCD images. The sensor surface is very delicate, and, unless you use film a lot, there is not likely to be too much contamination, but if you notice that the resolution of the CCD starts to deteriorate, the sensor may need cleaning. I would not recommend this procedure as a part of routine maintenance, and I would definitely let the professionals handle it. Bill Tivol william.f.tivol@aero.org Wed Dec 22

I don't have an online resource to point you to, but here are a few hopefully useful points that we typically give our customers: (1) Scientific microscopy cameras do not introduce an anti-aliasing filter into the system and therefore do not need cleaning of this element. (2) Scientific cameras usually have a front chamber window to keep dust out of the sealed chamber that houses the sensor. Simple sealed chambers are sealed against dust but not moisture and are used for non-cooled cameras. Stronger seals prevent both dust and moisture infiltration and are used on cooled cameras to prevent moisture condensation on the sensor. (3) Some cameras sensors are provided in a "windowless" package exposing the surface of the sensor to the environment present in the sealing chamber itself. This configuration of camera should only be considered for the most demanding of applications due to the risk of sensor contamination. Cleaning of the sensor surface is only possible on non-micro-lens designs, and then only should only be attempted as a last resort as the surface and bonding wire connections are extremely delicate. (4) Cleaning should be limited to the outside of the sealing chamber window. Breaking the seal to the sealing chamber may result in dust particles being introduced into the chamber if not performed in clean hood environment. Additionally, breaking the seal on cooled cameras may result in moisture infiltration and saturation of the molecular sieve resulting in condensation on the sensor. Sarah Cosgrove cosgrove@diaginc.com Wed Dec 22

TEM:
alignment

I am looking for advice, instructions, warnings, etc. about attempting a full alignment on our ten-year-old JEOL JEM2010 with FasTEM. Something that goes far beyond basic alignments one does each day; information not in the instruction manual included with the microscope. Specifically, when should one use/not use free lens controls? How to correct image shift when magnification is changed? Why do select area diffraction pattern come from an area outside the region selected by the SA aperture? These, and other issues, I presume could be resolved if a complete alignment were done on a semi-annual basis. If someone has a description of what turning each of the alignment knobs actually does to each lens/coil that also would be very helpful. I am attempting to compile a table of these relations by observing lens current changes while turning each knob, but this has proved very tedious and imprecise. Why do I need this information? (1) We are a user facility with 20+ operators of various skill levels. (2) We don't carry service contracts on our microscopes so I can't call for help every time the beam gets lost. My institution is willing to pay for on-demand service when necessary, but I consider keeping a microscope "in tune" something that should be within the bailiwick of the on-site supervisor. ***Roger A. Ristau raristau@ims.uconn.edu Tue Nov 16***

Quite right, when done correctly and saved, the alignments on a JEOL TEM are a simple button touch (green N button under plastic cap, or computer recall) to recall and do not change much over the many months and I would say years. I am swamped right now but can try to help you off-line tomorrow. Free lens is never needed for regular use. Deflectors are what you need to track and set in a spreadsheet for referrals. If a battery to memory fails you may lose the values so it is wise to have them written down. Roseann Csencsits rcsencsits@lbl.gov Tue Nov 16

The simple answers to your questions are: For basic use of the microscope Free Lens Control (FLC) should not be used at all! FLC turns the microscope into an interesting optical bench but is not needed for standard use of the microscope. Image shift with magnification change is usually the result of condenser alignment issues and can usually be addressed by selecting the appropriate alignment on the right hand draw and returning the value to the last engineer set one by pressing the green N button on the right hand side of the draw. SAD patterns not coming from the same area as in Imaging mode is also probably the result of the same alignment being incorrect. To return all alignments to the last engineer setting select each in turn and press N. However I suggest noting all values down (preferably in hexadecimal—on FasTem server, select Maintenance > System Status > System Maintenance Page > Maintenance DA/C to put all DAC values onto Microscope Screen (select System Main page > P1 Main to get back to original setting)). All of the alignment coils controls the alignment coils in the column. Shifts shift the bean and DEF tilt the beam. Shifts apply equal but opposite current to the upper and lower coils while DEF apply different currents to upper and lower coils. Any deeper alignment than this really does need an engineer and has to be done outside of FasTem. Alan W Nicholls nicholls@uic.edu Tue Nov 16

The reality of TEM alignment or SEM for that matter is that there are basic procedures that are repeated to a conclusion to enable the column to be aligned to the level that you require. Just one caution people do spend too much time messing with instrument alignment; "if it is not broken don't fix it"! 1. One method requires repeating two actions with sets of balancing coils or a mechanical alignment. The most common procedure is that of aligning the gun and illumination shifts to bringing the double condenser system into alignment. In this procedure the gun shift is aligned when C1 is set on a large spot size and C2 is brought to focus. Then C1 is set at a higher current, C2 brought to focus and illumination shift used to obtain the alignment. The procedure is repeated until a common centre is achieved. A similar procedure may be used to align the last two lenses in the column; let's call them projectors. Center a feature in an image on the screen with the final projector; P2. Determine the point when the P1 starts to take part in the magnification process and centre the feature with the P1 alignment. Drop the magnification and centre P2 increase the magnification and centre P1 repeating until you have a constant centre. 2. Moving back up the column another common alignment method is to watch the image as the magnification is increased checking to see when the next lens up the column is being activated. You may see the flash of a diffraction spot which going up the magnification range is in one position. Moving down the magnification range note the second position of the diffraction spot. Having judged these two positions move the lens (Intermediate?) to place the centre point between these two spots on the centre of the screen. 3. The simplest method for bringing the image centre on axis throughout the magnification range is to take note of which lens is changing as you run up the magnification. Image shifts occur as the emphasis moves from lens to lens as the magnification range develops.

When the selected image point takes a shift check on the lens now contributing and move the image point to the column axis with that lens alignment facility. 4. As for movement between selected area mode and diffraction the most common error is not applying the rule correctly. In selected area mode the operator should use the freed up Intermediate lens to focus the selected area aperture edge. Once the aperture is in focus then the specimen must be brought to focus in the normal way. This double action brings the image focus to be in the same plane as the aperture, which should result in a true representation of this area when moving to diffraction. 5. Illumination shift when changing focus is rarely an alignment fault! The lens field from the very strong objective current overlaps the weak second condenser field and dominates; this results as a shift. Hitachi tried to overcome this through the top hat pole piece design reducing the field overlap. JEOL for many years simply had a deflection coil that deflected the beam to compensate for an objective current change and illumination shift. Philips (now FEI) compensated for the illumination shift due to this overlap by an alignment of the two objective pole piece units. 6. Most instruments change the final projector through the very lowest part of the magnification range; usually recognized by strong image rotation unless they compensate with a lens higher up the column. On some occasion one lens is providing magnification whilst another lens is demagnifying, all because it is best to use a strong lens even at low magnifications. Just one observation, having watched many hundreds of people set up an instrument; only spend an appropriate period of time aligning an instrument. Working at high magnification for a long period of time put effort into the alignment. For just a quick look-see, then only spend minutes on the alignment. I often compare the "urge to align" with letting the air out of your car tires each morning and then re-inflating them ready for the day. If the microscope was aligned yesterday it will be aligned today. Finally, keep a note of the lens currents at steps throughout your magnification range and a note of "normal" deflection coil settings also helps. Steve Chapman protrain@emcourses.com Wed Nov 17

TEM:
nanoparticles

For those of you who routinely image nanoparticles in your TEM, what sample preparation tricks do you use to prevent the nanoparticles from becoming contaminants to the movable and fixed apertures? ***Randy Nessler randy-nessler@uiowa.edu Thu Nov 18***

We do a lot of nanoparticle work, mostly with polymers but also with metal and ceramics. I've not found contamination of the apertures to be much of a problem; the vast majority of our work is done in cryo conditions so the targets are neatly trapped in water ice. For room temperature work all our stuff is applied as an aqueous suspension then blotted or allowed to dry, as long as no one puts in grids so heavily loaded that bits are flaking off we don't get problems. I've once had a problem with silica spheres that charged up and scattered under the beam, a few did make it to the apertures but simply cycling the aperture in and out a couple of times was sufficient to dislodge them. Cheers Ian i.j.portman@warwick.ac.uk Fri Nov 19

I've only looked at nanoparticles in cryo, and we assumed that too few of them would leave the grid to be a contamination problem. I am pretty sure that this is a good assumption for the areas of the grid that had minimal or no exposure to the beam, but it may not be so good for the areas that were imaged. On the other hand, the scope was not located in a clean room, so any particles in the ambient environment could make their way into the airlock and then into the scope. Bill Tivol william.f.tivol@aero.org Fri Nov 19

TEM:
measure electron dose

I look at some biological materials in a JEOL 2100F with a Gatan Tridiem GIF camera. The problem I have is to figure out the electron dose. The viewing screen gives the value of the current, but I am not sure how accurate this number is. I was wondering if there is a Faraday cup in the GIF system with which we can measure the current. Does anyone have an idea on this, or a manual of the GIF system? ***Yifeng Liao liaonu@gmail.com Fri Nov 19***

When we had this problem on our scope, the service engineer calibrated the screen current using a Faraday cup. After that, we could rely on the screen current measurement to calibrate the CCDs, which was done each month. Then we could calculate the dose for the magnification we wanted in terms of the counts/pixel on the CCD. There is a way to have the beam hit the drift tube in the GIF, but that might not be an accurate absolute measurement, although it can be calibrated with the Faraday cup at the same time as the screen. Bill Tivol william.f.tivol@aero.org Fri Nov 19

We regularly use the GIF drift tube as a Faraday cup to measure the probe current. The Drift Tube connector is one of the BNC-type connectors near the neck of the GIF. Ours is the middle one of the three for the GIF 200 and GIF2000 (it is worth checking the schematics of the GIF connections). Switch off the Gatan Instrumentation Bin (GIB) and connect coaxial BNC lead to a pico-ammeter. You'll need to focus the beam down as small as possible and use the beam shifts (imaging mode) or diffraction shifts (diffraction mode) to steer the beam into the spectrometer. If you need to, I can provide some photos of the arrangement next week when I get hold of the pico-ammeter. If you use a swivel chair with your microscope, you can see (& measure) the induced current in the GIF by moving around on it! A good way to see if the chairs you use are incompatible with operating GIF, especially for sensitive spectrum measurements! Jon Barnard jsb43@hermes.cam.ac.uk Fri Nov 19

TEM:
changing oil

Does anyone know the protocol for changing oil in the Pfeiffer-Balzer 170 THP series turbo pump used on the Zeiss 902 TEM? I have the TL 011 oil and I know that each side gets 20 mls, but do you pour it in, force it in or is there a trick. It just seems to not to what to come out or go back in easily. ***Bill McManus bill.mcmanus@usu.edu Mon Dec 20***

I was working on a DSM950 SEM that had a 170 and called Pfeiffer. It seems a little counter-intuitive, but they told me to just pull the pump out, lay it on its side with the plugs out of both sides of both bearings and pour the oil through. Most of it just flows out (flushing the innards) and what remains is what you need. I still don't feel real comfortable with that procedure, but it was straight from the Pfeiffer service department. Ken Converse kenconverse@qualityimages.biz Mon Dec 20

I'm with Ken on this. The horizontal bearing TPH-TPU pumps need quite careful attention. The caps are removed taking care to note the springs that push the wick assemblies onto the end of the shafts. The oil is then syringed out using a large syringe with a bit of silicone tube on the end. A new charge of oil is then introduced with a new clean syringe. My manual says 10 cc. Do not overfill. The oil is conducted to the bearings by the wick and the bearings are not immersed in oil. Carefully replace the end caps making sure that the springs are correctly positioned and not kinked to one side. Kerry Gascoigne kerry.gascoigne@flinders.edu.au Mon Dec 20

This is not the horizontal "dual" pump (TPU 330). It's a vertical one, but I was told to just lay it on its side and run the oil through it. I agree with your tips on the 330. Ken Converse kenconverse@quality-images.biz Mon Dec 20

I confirm what Ken told. Put the pump (unmounted from the SEM) horizontally on two wedges, unscrew the pairs of bright slit screws mounted in opposite, one in the middle of the pump, the other at the motor side. I have a funnel sold by Pfeiffer, which is to be screwed on place of the upper screw, with a mark at the oil level. It's useful to avoid oil leaks at the funnel, as the holes are small, but one can do it with a very small funnel, or a little tube connected to a funnel. Poor the quantity of TL11 oil in the funnel and it flow slowly trough the wick, which is around the ball bearing. No pressure at the inlet, only gravity! Keep care at the beginning to let loose some bubbles which can block the flow at the inlet with a piece of piano wire. Put a small dish under the lower screw hole, to collect the dirty oil. You'll see the oil coming out first very dirty, and becoming more and more clean, until you have collected a little less then what you have poured in. It takes some time (1/4 h for each bearing), as you must wait until it has almost finished to drop. Same thing for both ball bearings and it's good for 1 year (in continuous use). By the way, the old oil is very fine for your bicycle chain, or other such uses! Jacques Faerber jacques.faerber@ipcms.u-strasbg.fr Tue Dec 21

Along with the TEM came a detailed instruction brochure from Pfeiffer; if you can't find it contact Pfeiffer for a pdf or me offline. Changing oil is absolute simple and easy, but you need the small white funnel supplied with the pump. Peter Heimann peter.heimann@uni-bielefeld.de Tue Dec 21

TEM:
Schertzer focus

I have been reading that most microscopes can be preset on "Schertzer focus" and there is discussion of "optimum underfocus." There are also significant changes in contrast associated with changes in focus that can lead to misinterpreting images. ***Bernard Schreurs bschreurs@brni.org Fri Dec 10***

First, I recommend that you read any good reference book on transmission electron microscopy. The section on phase contrast microscopy will describe Schertzer focus—if there is no such section in the book you have chosen, look for another book. A simple definition of Schertzer focus is that it is the point where the effects of spherical aberration and defocus offset each other to the maximum extent (this always happens at underfocus). This allows information to be transmitted from the specimen to the image in phase at the highest spatial frequency for which there are no phase reversals at lower spatial frequencies. High spatial frequency corresponds to small details, and the lack of phase reversal means that interpretation of the image is pretty straightforward (there is still some loss of intensity at high spatial frequency, that is, a loss of contrast for small details, but bright things stay bright and dark things stay dark). Optimum defocus (also always at underfocus) is where the effect of spatial incoherence (the effect of a source of electrons that is of finite size) of the beam is minimal. This means that the envelope function, which damps out contrast, is as constant as possible to high spatial frequencies. In practical terms, this means that the signal-to-noise ratio is highest for details of all sizes down to the smallest resolvable. In other words, it is the defocus for which details of all sizes are equally visible (as nearly as possible). For phase contrast imaging, the information in the specimen that is transmitted by the microscope to the image can either be in phase or out of phase. In the latter case, the appearance of the image will be different from the specimen, giving the possibility of misinterpretation. One can compensate for the out-of-phase character of the information at spatial frequencies where the phase is reversed by dividing out the effect of the contrast transfer function—look it up in the book—to get a more simply interpretable image. Bill Tivol william.f.tivol@aero.org Fri Dec 10

TEM:
jewel pivot pins

Does anyone know of a source for the small jeweled pivot-pins used to hold specimen cup on double-tilt TEM holders? It seems that heavy-handed users in our multi-user facility break these jewels every year or so, and I am not satisfied with the hassle of sending out the holder for repair. I am sure we are not the only ones to experience this. Does anyone have hints for on-site repair? ***Roger A. Ristau raristau@ims.uconn.edu Wed Dec 8***

We have some JEOL double tilt holders, which have jeweled pivot screws and bearings on the tilt gimbal. We have had experiences of these being damaged and we do repair them ourselves, obtaining the pivot screws from JEOL. There are several causes of user damage that are avoidable. It is important that the holder tip is lowered gently onto the support after the heavier end has been put down fully and that the tip is lifted first. This will avoid the gimbal taking the weight of the holder on the delicate pivot jewels. It is important to ensure that the holder tip is sitting straight on the stand, that the support is directly below the gimbal and that the holder is not rotated on the stand. Later support stands do square up the holder to ensure it is not rotated. The JEOL holder stand has a variable height centre support that is set at the factory but I have known these to become loose and users to adjust them incorrectly so that the weight of the holder is being held by the gimbal as opposed to the weight of the holder being taken by the stand with the gimbal support just underneath the gimbal and not taking any weight. Users can try to overtighten the specimen screw ring, which will rotate the gimbal and can break the pivot screws. This is a matter of 'feel' and there are a few people who are unable to be delicate, they ought not handle delicate items and it may be better to make alternative arrangements for their specimen loading. It is best to take the above precautions and not to damage the holder in the first place but we have had enough problems with our holders that we now keep pivot screws in stock and replace them in-house. The screws are not cheap but we can get a holder back in action in a day or so provided that the bearing cup on the gimbal has not been damaged, we have not been able to source the bearing cups and have to replace the gimbal complete. Apart from the delicate nature of the repair work it is relatively simple, basic instructions from memory are: Carefully unclip the gimbal from the tilt mechanism so that the gimbal is free to rotate. The original screws will have been sealed with epoxy resin to ensure that do not move from their set position. This must be dissolved with acetone before you can remove the old screw. We use a cotton bud to keep acetone only on the screw to be removed. When the screw has been removed the tapped hole needs to be cleaned of any epoxy remains. Fit the new screw such that the gimbal will just not drop under its own weight then back off the screw 5–10 deg rotation so that the gimbal is free to drop under its own weight but there is no side to side movement of the gimbal. Put a little, and I mean a little, epoxy onto the top of the screw to keep it in the set position. Do not get it into the screw thread or it will be very difficult to remove for any future repairs. Replace the gimbal into the tilt mechanism. Check that the holder stand is still set to the correct height for the new screw(s). Leave the epoxy overnight to harden before exposing it to the vacuum system. If the above does not seem obvious to you

then is it probably best that you do not attempt the repair. We have been unable to source the pivot screws except from JEOL but we have developed a method of removing the old jewel from the screw and have made a new screw from one with a damaged jewel and one with a damaged screw head. We have managed to source the jewels separately but we would have to buy them in bulk; I think it was 20 minimum so we would have to make several pivot screws for it to be economically viable. We hope not to have to repair that many holders. Ron Doole ron.doole@materials.ox.ac.uk Sat Dec 11

In regard to Roger Ristau's and Ron Dooley's discussion of the replacement of damaged jeweled pivot screws in double-tilt specimen holders: I wonder if the use of jeweled screws in the first place is not a bit of an overkill. These devices do not rotate very rapidly, nor through great amounts, and so I would think that carefully polished metal pivot screws would serve as well. These could be made rather easily by most machinists, and they would not be brittle and easily broken. Wilbur C. Bigelow bigelow@umich.edu Mon Dec 13

When I was in graduate school, my advisor, John Hren, told me that his group had made some special TEM rod holders. They fabricated the jewel surface by taking a glass rod and heating it and stretching it so that it was very thin. Then after they broke the glass strand, they heated the end and it made a glass sphere that they could control the size by how much they heated the end of the strand. When they got the size that they wanted, they broke off the sphere. The little tip of glass that was attached to the strand went into the hole in the end of the rod and they glued (epoxy??) it in the end of the rod. He said it worked well. I made a rod for FIM samples and used the technique on a holder for a JEOL 200 CX. Scott D. Walck swalck@southbaytech.com Tue Dec 14

SEM:
absorbance current variation

I have a problem with my SEM equipment marked by an absorbance current variation after filament exchange (good centered), with a value only of 20 pA at PC10 and big variations (from 20 to 600 for example) at scan rate 6 or greater or magnification greater than 1000×. So these variations modify my images by changing the contrast and take place in the same time with the absorbance current variation. I clean most of the metallic parts (anode, cathode or apertures or supports) and have no improvement; under 5 scanning rate the image doesn't vary but the image is not very good. I think that can be an electrical problem but for reduced amplifications or a scan rate of less than 6, the images obtained are fine. Please give me some clues about what I should re-check or what can be the problem. ***Nicanor Cimpoesu nicanornick@yahoo.com Fri Nov 19***

We probably need some more information. Are you having problems with faster or slower scan rates? Have you checked the absorbed current behavior in a Faraday cup with different magnifications and scan speeds? What is your sample? What kind of SEM are you using and under what conditions? I'm leaning towards sample charging if the problem is worse with higher magnifications and slower scan speeds. There won't be any problem if you check the absorbed current with a Faraday cup. Some samples, such as quartz grains, will charge even though they are coated because the quartz is an excellent insulator. The coating will carry off any surface charge, but a 20 or 30 kV beam will inject a substantial charge several microns into the grain, where it will remain for long periods of time, unless the sample is brought to atmosphere. There are many other types of samples that can have similar issues. Try changing your accelerating voltage and condenser lens setting (spot size) and see if things change. Ken Converse kenconverse@qualityimages.biz Fri Nov 19

I don't have now a Faraday Cup but I will get one Monday morning and try to analyze the problem, I don t analyze a specific material, I use the normal stub provided with microscope, the model is Vega Tescan LMH II, and I am at 500 hours usage of the microscope and 3 hours of new filament (by from a Tescan delivery brand from Romania—so should be trusted). I have my absorbance current variations at high scanning rates (more than 4) when I try to obtain a proper image of a metallic material. The problem persists at 5, 10 and 20 kV filament power supply and the manual centering of the gun doesn't give any results. Nicanor Cimpoesu nicanornick@yahoo.com Fri Nov 19

You mentioned using a metallic specimen. That could be about as good as a Faraday cup, assuming it is not oxidized. I would also consider a piece of graphite. We have plenty of that from rods used for carbon evaporation. We have also used graphite to make specimen stubs. Drilling a hole partway through the graphite and centering that under the beam would be a first attempt at a Faraday cup. You could also fasten an aperture over the top of the hole with some conductive paint. That would make it a very effective cup. I am confused as well why this would be a problem at fast scan rates. Charging should be less of a problem. Are you seeing bands of changing brightness as the beam scans down the frame? That would indicate some instability. Maybe it is fast enough that it only shows as random noise at slow scans. Maybe you can post a picture out there. I would be willing to receive one directly, but they cannot go through the list. Are all other conditions the same? Be sure that you have not accidentally gone to a much higher or lower beam current using a different aperture. Could the filament be unstable? Do you have another spare filament that you could try? Maybe the first one is wandering during these early hours of use. Maybe it is a rare bad filament. That would be unlucky, but possible. Warren Straszheim wesaia@iastate.edu Fri Nov 19

It is strange, that you have seen instabilities at high scanning rate only. This seems to be some high frequency noise (which gets averaged at low scanning rate). Have you performed automated heating and centering of the new filament after the replacement? What is actually your heating current and emission current? In my opinion, there might be several other reasons for filament instability: 1. Filament centering screws (which hold the filament inside the Wehnelt cylinder) are loosened. 2. Filament is damaged (e.g. broken wire) and its emission current is low—try another filament. 3. Wehnelt cylinder is dirty—the first filament lifetime was 500 hours—probably it is necessary to clean Wehnelt cylinder around the central hole. The problem of the sample should be definitely solved by using the metal sample (e.g. an empty sample holder) or Faraday cup—by the way, there should be two of them on your stage (see "Stage control" window). By the way, in a case of such problem, do not be afraid to contact Tescan support (support@tescan.cz) and attach package of microscope log files. Tomas Hrncir tomas.hrncir@tescan.cz Mon Nov 22

SEM:
calibration standard

We frequently use SEM (Hitachi 4700, FE) to measure some wafer cross-section features. The measurement result is sometimes off the mark. I wonder if someone here could recommend some fine grid (0.1 μm) standard. ***Jun He junhe1970@gmail.com Tue Dec 21***

I use a TEM carbon grating replica of a 2160 lines per mm cross grating. This gives a 0.4629-micron spacing and, if you fix it down carbon side up, the sample makes a nice example of the effect of kV on imaged information. Steve Chapman protrain@emcourses.com Tue Dec 21

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