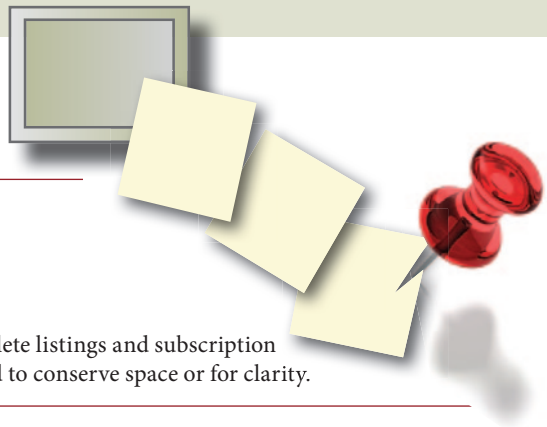


NetNotes

Edited by Thomas E. Phillips
University of Missouri
phillipst@missouri.edu

Selected postings to the Microscopy Listserver from March 1, 2012 to April 30, 2012. 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:
fixation

I note a strange behavior during a LM fixation and I would appreciate your feedback. I prepared my fixative as follows: suspended 2 grams of a 6-years old paraformaldehyde powder in milliQ water—Heated at 60°C for 10 min with magnetic stirring—Added 3 drops of 1 N NaOH to depolymerize—let cool down, then doubled the volume with 100 mM HEPES, pH 7.2. The final solution is 4% paraformaldehyde in 50 mM HEPES, pH 7.2. I used the solution 2 hours after preparation on cells grown on coverslips. I washed them just once with PBS before adding fixative. Under the LM, I can see impressive blebbing of the cell membrane and that's my concern. It seems that my solution is hypoosmotic, is it the case? In this hypothesis I could just add some NaCl to make it isotonic. At the bottom of my memory I seem to remember that iso-osmoticity should be approximately 260 mOsm. The problem is that I don't know how to take account of the presence of formaldehyde for the calculation of the osmolarity. At the beginning of the fixation I suppose that only a few molecules cross the membrane but as the fixation proceeds, more and more molecules cross the membrane. 4% is quite high so if it counts I guess that the solution is not hypoosmotic. ***Stephane Nizets nizets2@yahoo.com Wed Apr 11***

Blebbing in formaldehyde is a well-known phenomenon. I think Elizabeth Hay's group may have been the first to describe. Adding a small amount of glutaraldehyde can prevent it from happening. Formaldehyde penetrates quickly but reacts more slowly than glutaraldehyde. Osmolarity is only one factor. You are permeabilizing the membrane and allowing calcium and other electrolytes to cross. The only real solution is quick-freezing. Thomas E. Phillips phillipst@missouri.edu Wed Apr 11

From what I recall, the PFA doesn't really play into the osmolarity because it penetrates the cells so rapidly (although crosslinking takes longer). I have read where aldehydes can cause membrane blebbing visualized at the TEM level, but I agree with you on the hypoosmotic possibility. If your sure of the PFA's efficacy, (check shelf-life with manufacturer), try adding 1–3% sucrose in your fixative to boost osmolarity. We also add 3 mM $CaCl_2$ or $MgCl_2$ (for PO_4 containing buffers) to reduce enzymatic activity on the membranes (occurs even during fixation) for tonicity. I would also compare a different buffer like 0.1 M PO_4, even though the HEPES (how old is this buffer?) sounds fine. Michael Delannoy mdelann1@jhmi.edu Wed Apr 11

I use 10% of the formaldehyde molarity and 30% of the glutaraldehyde molarity and add that to the buffer. This seems to work well. The formula comes from page 66 of "Biomedical Electron Microscopy" by Maunsbach and Afzelius. This is a book that I think very highly of, but have no financial interest in. This formula has served me well through the years. David Elliot dae1@email.arizona.edu Wed Apr 11

Most answers point out that swelling of the cells is a known effect of formaldehyde fixation and that increasing the osmolarity of fixative could be favorable. Some say that the concentration of formaldehyde should not be taken into account for any osmotic effect; one person uses only 10% of the formaldehyde concentration for the calculation. Now a new but related question: I have found a 6-years old bottle of 37% formalin which was already open (but maintained closed) and stored at room temperature. Do you think it is still good for basic immunofluorescence work? I just want to stain one cell type in a mix culture so it is not high precision work here. Stephane Nizets nizets2@yahoo.com Thu Apr 12

Thanks for at least resuming about other answers you got off-list concerning your first question. I think it is worth to know about the "differences" between "osmolarity" and "tonicity" (which is/has been discussed not only once, also on this MSA listserver). Concerning your "new" question: You don't say anything about brand or storage conditions of the bottle of 37% Formalin (guessing—for Austria—it might be the Merck formalin 37% stabilized with about 10% methanol and calcium carbonate for histology. The stabilizer methanol as well as the added calcium carbonate function in prevention of polymerization and as buffer medium for generated (formic) acid. Usually the product had imprinted an expiration date on the label, which isn't always followed today, e.g. a fresh bottle of that product was purchased in 2010-10 and show(s)/showed an expiration date 2012-05. Assuming you can see that expiration date—under certification rules—one can/is allowed to use the content until 2012-05 without any consequences. If you/your lab aren't/isn't subject to such regulations I would consider the use of that charge/lot of fluid (if storage was in agreement with the suppliers data) until End of 2012 is o.k. After that date one could use the solution for fixation e.g. in Routine Histology/fixation of corpses etc. Last but not least: you don't know about the real concentration as well as about the grade of polymerization in your old solution. Knowing that filtering does not really withdraw polymers of formaldehyde, one should be careful in pipetting or spilling the solution out of the bottle . . . latter perhaps may churn up the calcium carbonate powder which perhaps interferes with your microscopical preparatory intentions. Wolfgang Muss w.muss@salk.at Thu Apr 12

Specimen Preparation:
bacteria for TEM

Can anyone explain how to prepare bacterial cells for TEM observation? The literature I am reading mentions adding molten agar to the fixative. I have never come across agar in our lab. Can anyone comment on that? Is it ok to go from the fixative to the wash to the fixative? ***Ashley Rodriguez arodriguez334@students.deltacollege.edu Wed Apr 25***

If you do not have agar in your lab, I assume the bacteria have come from somewhere else because agar is the standard growth medium. You don't necessarily have to encapsulate the bacteria in agar it is often just a convenient way of handling a pellet of small cells without them breaking up during fixation, dehydration and embedding. I have only used it if I needed to retain the structure of

https://doi.org/10.1017/S1551929512000466 Published online by Cambridge University Press

a microbial colony intact or because the cells were likely to disperse. Most samples of bacteria can just be spun down in a microcentrifuge between each stage of fixation to retain a pellet. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Thu Apr 26

Using molten agar for bacteria sample preparation is quite simple. General rule is to mix equal volumes of bacterial pellet and molten agar. After agar solidification, the agar-bacteria gel is cut into the small cubes (approximately 1 mm × 1 mm × 1 mm). The cubes are then processed as pieces of tissue. The advantage of this is that you do not need to centrifuge the bacteria sample between next processing steps. In our lab, we use a low-melting agarose instead of agar. Our common procedure is following: A: Make a 4% low-melting agarose in washing buffer or ddH_2O in an Eppendorf tube. It can be stored for some time in a refrigerator. B: The procedure 1. Fix the bacteria sample (in Eppendorf tube). 2. Wash the sample well (at least three times). 3. Liquefy 4% agarose gel and temper it to 30°C or 37°C in water bath. 4. Gently spin down the washed bacteria sample. 5. Remove as much as possible of the supernatant. 6. Estimate the volume of the sediment. 7. Add an equal volume of 4% agarose and gently mix (after this step, you can gently spin down the mixture). 8. Let solidify the mixture. 9. Cut off the bottom of Eppendorf tube containing the agar-bacteria gel. 10. Take the agar-bacteria gel out and cut it into the small cubes with a razor blade. 11. Postfix the cubes in OsO_4. 12. Process further as common tissue block (wash, dehydrate, embed into resin, etc.). Oldrich Benada benada@biomed.cas.cz Thu Apr 26

Specimen Preparation:
Formvar vs. Butvar

I would appreciate hearing about the differences between Formvar and Butvar. How do they compare? What motivates using one vs. the other? Tobias ***I. Baskin baskin@bio.umass.edu Wed Feb 29***

Butvar is purported to be more hydrophilic, more adhesive, stronger and more translucent. Tom Phillips phillipst@missouri.edu Fri Mar 2

Pioloform is much stronger in the beam than Formvar or Butvar. It is soluble in dichloroethane or chloroform. You should add molecular sieves to trap any water molecules to give you films with less hole defects. You can prepare films as you do now with Formvar or Butvar. Formvar and Butvar, so last century. Rick Fetter fetterr@janelia.hhmi.org Sun Mar 4

Which Pioloform do you mean? Formvar and Butvar are trade names for polyvinyl formal and polyvinyl butral. I believe that Pioloform is a competing trade name and comes with various letters. For example, Pioloform B is I think the same chemical as Butvar. I am pretty sure you can get the Pioloform version of Formvar too. So which Pioloform are you recommending here? Tobias I. Baskin baskin@bio.umass.edu Sun Mar 4

Specimen Preparation:
staining for inorganic particles

We have negative staining for nanoparticles coated with long chain unsaturated molecules, biological samples, etc. Can we also have some kind of staining for the inorganic shell of nano particles. For example, if one metal is coated on another can we selectively bind some ligand to outer shell etc to get better resolution? Has anyone tried such a thing? ***Amit amit.welcomes.u@gmail.com Thu Mar 8***

If I understand your question correctly, what I think you are asking is if you can enhance the appearance of the nanoparticles so you can see them better in the microscope. With certain particles, the answer would be, yes. It is possible to do silver or gold enhancement of metal nanoparticles to make the particles "grow" or become larger, chemically. This is a common procedure done in immunogold labeling. There are kits sold by the various electron microscope supply companies for this very procedure. The technique is fast and easy to do, normally done with gold particles. By varying time of exposure and/or temperature, you can end up with different size nanoparticles. Check with the vendors to see if this type of enhancement will work with the kind of nanoparticle that you are working with. Edward Haller ehaller@health.usf.edu Thu Mar 8

Suppose I have a core-shell particle of an inorganic core such as silicon carbide and over it is thin shell of SiGe or Fe_2O_3 with shell of MgO. Can I enhance the contrast between the chemical moieties? I read one paper where the group was doing the same with negative staining of gold nano particle coated with (if i remember correctly) PEG. Amit Gupta amit.welcomes.u@gmail.com Thu Mar 8

PEG can be stained positively with uranyl acetate stain. In other words, it will stain with uranyl acetate. It is not a negative stain, but a positive stain. PEG will get dark when exposed to uranyl acetate stain. The other alternative, as I mentioned, would be to grow the gold particles through silver or gold enhancement. Your silicon, magnesium and iron particles are already electron dense. I would be working at 60–80 kV for this work, 100 kV maximum. I'm taking for granted that you're not trying for lattice resolution on anything here. Higher kV will not give you the contrast that you want. Use a 50 micron aperture for your work and you should have all the contrast you want. Edward Haller ehaller@health.usf.edu Thu Mar 8

Another possibility no one else has yet mentioned is to evaporate a metal onto the specimen; e.g., platinum shadowing. If the specimen is high-Z, it will be difficult to distinguish the shadow from the shell, but energy filtering could do that. In any case, the resolution is unlikely to be improved by staining, since it will be limited to the size of the stain particles. There are high-resolution techniques for depositing a layer of Cr that are used in HRSEM, so maybe you could try something like that. Bill Tivol wtivol@sbcglobal.net Thu Mar 8

Specimen Preparation:
chemical mechanical polish damage

During the Si wafer growth and preparation process, how much damage is produced from the CMP process? Also, I've read that an epitaxial layer is grown on the wafer by passing silane gas over the surface. Will someone comment on the way these processes impact the structure of the Si at the surface of the wafer? ***Marissa mlibbee@gmail.com Mon Apr 2***

CMP means Chemical Mechanical Polish. That means that the silicon is lightly etched while it is being polished. Any amorphization is so little that it could not be detected by TEM. It is almost ready for growth of gate oxide at this point. The epitaxial layer grown with silane is usually not pure silane, but accompanied by some controlled impurities to dope the surface of the wafer. You will never see any evidence in a TEM of the interface between the epitaxial layer and the underlying wafer in semiconductor quality silicon. The only thing you may see at the surface of either CMP or epitaxial silicon would be a thin layer of oxide, called native oxide. The thickness can depend on how long the silicon was exposed to air and what other species may be adsorbed onto the surface. The native oxide would generally be only a few angstroms thick. John Mardinly john.mardinly@asu.edu Tue Apr 3

Could grazing-incidence electron diffraction, used, for example, by Larry Marks (sp?), detect and/or quantitate surface changes produced by CMP and silane? Bill Tivol wtivol@sbcglobal.net Tue Apr 3

https://doi.org/10.1017/S1551929512000466 Published online by Cambridge University Press

Bill, At that point it becomes a research project. John Mardinly John.Mardinly@asu.edu Wed Apr 4

Marissa asked about characterizing surface changes on silicon wafers as a result of CMP and epitaxial deposition. John Mardinly and Bill Tivol commented regarding the general characteristics of the materials, especially the ultrathin films involved, and briefly discussed TEM and grazing incidence electron diffraction. I have had great success in looking at silicon surfaces with the AFM (atomic force microscope). It is exquisitely sensitive to small height changes, down to the atomic scale. An example AFM image of an epitaxial layer is shown at http://www.asmicro.com/corporate/yale.htm. AFM is the tool of choice in the semiconductor industry for characterizing surface changes. It is easy to see scratches and to measure surface roughness down to RMS roughness <0.1 nm. Don Chernoff donc@asmicro.com Wed Apr 4

Specimen Preparation:

carbon contamination in plastic bag

Can a semiconductor or dielectric material be contaminated by carbon by storing it in a plastic bag? Can it influence [C] measurements in the ppm range? Any experiences and thoughts out there? ***P. Baggethun pal.baggethun@elkem.com Wed Apr 25***

From our experience, the answer is yes! Plastic boxes or bags will outgas solvents remaining from their manufacturing. I've no quantitative data, only the conclusions of studies made by a colleague here, now retired, made 20 years ago. His conclusion was that, if the surface cleanliness of a sample is important, it must be stored only in clean (plasma treated, at that time by glow discharge) glassware. Never plastic. I also observed such contaminations, but cannot share more than qualitative observations. On the other hand, the semiconductor industry uses most plastic boxes (fluoroware). They cost a lot. Maybe someone from that domain has more information? Jacques Faerber jacques.faerber@ipcms.u-strasbg.fr Thu Apr 26

Immunocytochemistry:

multiple labels with immunogold

I have a client who hopes to do triple immunogold labeling. I have been considering ideas including peroxidase-DAB or peroxidase-ENZMET for one of the labels and 2 sizes of gold for the others. I thought that I would toss this out as an intellectual exercise to those of you who do this all the time. Here is another complication: she has 3 primary antibodies and all are available from rabbit or goat hosts, but that is it. Two of the primaries will have to be from the same host. I thought that I would try Protein A or G for one of them, using excess, untagged protein to block before the second primary is used. Long and tedious. Any better ideas? I know I can count on the listserver for suggestions. ***Lee Cohen-Gould lcgould@med.cornell.edu Fri Apr 20***

There are two issues in your question: (1) can you resolve three sizes of gold; and (2) what antibody scheme do you need avoid cross-reactivity? There are papers describing triple labeling with 5, 10 and 15 nm gold—for example: Wakayama, Y.; Inoue, M.; Kojima, H.; Murahashi, M.; Shibuya, S., and Oniki, H., "Localization of sarcoglycan, neuronal nitric oxide synthase, beta-dystroglycan, and dystrophin molecules in normal skeletal myofiber: triple immunogold labeling electron microscopy," *Microsc. Res. Tech.*, 55, 154–163 (2001). If you don't want to go this large, I would suggest detecting the first target with conventional immunoperoxidase with DAB—this gives a diffuse signal that is easily differentiated from the particulate signal from gold (and DAB can be silver-enhanced for greater contrast, or you could use a signal amplification method such as ABC); then you only have to differentiate two gold labels, so you can label the remaining two with different sizes of gold—or one with small gold plus silver enhancement and one with 5 or 10 nm colloidal gold (much as we would like to see a good EnzMet EM application, this is probably not the place). Alternatively, you can use Nanogold with differential gold enhancement: Paspalas, C. D.; Perley, C. C.; Venkitaramani, D. V.; Goebel-Goody, S. M.; Zhang, Y.; Kurup, P.; Mattis, J. H., and Lombroso, P. J. *Cereb. Cortex.*, 19, 1666–1677 (2009). Paspalas, C. D., and Goldman-Rakic, P. S., "Microdomains for dopamine volume neurotransmission in primate prefrontal cortex," *J. Neurosci.*, 24, 5292–5300 (2004). That now leaves the problem of three targets with two hosts for the primary antibodies. You could use a biotinylated secondary antibody and labeled streptavidin as a tertiary probe for one (suggest the first one, detected with HRP-DAB), and block afterwards with excess unlabeled secondary if necessary. Then the other two targets can be treated as a double labeling experiment, making sure that the labeled secondaries are pre-absorbed against the host animals for the other antibodies. Rick Powell rpowell@nanoprobes.com Tue Apr 24

Ralph Albrecht at Wisconsin was (is?) working on colloidal probes with different morphologies (triangles, faceted, etc.) to provide distinguishable BSE signals. Some of the probes were pretty big, but a number of them were <15–18 nm. Ref: Meyer DA, Oliver JA, Albrecht RM, "Colloidal palladium particles of different shapes for electron microscopy labeling," *Microsc Microanal* 16(1):33–42 (2010). Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/20030909. Aaron Barnes barnesa@umn.edu Tue Apr 24

In the approach your described, I assume the purpose of blocking with unlabeled secondary after the first labeling using immunoperoxidase (ABC system) is to block the epitopes on the first primary (e.g. rabbit primary I) that are not bound by the first anti-rabbit secondary (biotinylated). If that is the case, I think the followings are potential issues. 1. Since the primary against one of the remaining targets (e.g. rabbit primary II) is from the same species as the first primary (rabbit primary I), rabbit primary II will bind to free Fab from the first secondary as well as to the unlabeled secondary used for blocking. 2. Since two anti-rabbit secondaries are from different sources, they probably are against different set of epitopes on rabbit primary. This means that the second anti-rabbit secondary could still bind to the first primary even with the blocking using unlabeled secondary. I remember reading literature years ago that the reaction product of the HRP could mask the antibody binding sites, although I have never tried it myself. If that is the case, the HRP enzyme reaction may render the first anti-rabbit secondary unavailable, which is helpful in reducing cross-binding. Similarly, silver enhancement of ultra-small gold conjugates also have the effect of encapsulating the antibody therefore double labeling using primaries of the same species can be done sequentially (K. Bienz, "Electron microscopic immunocytochemistry. Silver enhancement of colloidal gold marker allows double labeling with the same primary antibody," *J Histochem Cytochem*, Oct. 1986, 34(10): 1337–42). Hong Yi hyi@emory.edu Wed Apr 25

Yes—blocking the first rabbit primary would be best done by using the unlabeled secondary against the second rabbit primary, thus covering epitopes to both secondaries on the first primary. However, you could help avoid the second issue (rabbit primary II binding to free Fab from the first secondary) and simplify the process, by using the same secondary antibody for both rabbit primaries—but use biotinylated monovalent Fab fragment as the secondary against rabbit primary I, then gold-labeled IgG from the same host species as the secondary against rabbit primary II—thus labeling and blocking rabbit primary I at the same time. I should clarify that

https://doi.org/10.1017/S1551929512000466 Published online by Cambridge University Press

the scheme I suggested needs an intervening wash between the first primary and blocking, and the subsequent primaries; I don't see a way to get around that. Apply rabbit primary I then biotinylated secondary antibody (the streptavidin-peroxidase could be applied next, or later), then wash thoroughly, then apply the primary against the second (or primaries against the second and third) target(s). The sequence of steps might be: (1) Rabbit primary I against the first target. (2) Biotinylated Fab donkey anti-rabbit secondary (Jackson Immunoresearch). (3) Streptavidin-peroxidase or ABC, develop; or Nanogold/small gold-streptavidin, then silver or gold enhancement. (3) Wash thoroughly. (4) Goat primary plus rabbit primary II against second and third targets. (5) Secondary 6 (or 12 nm) gold labeled donkey anti-goat for the second target, and 12 (or 6 nm) donkey anti-rabbit (Jackson) against second and third primaries. Jackson's donkey anti-goat is available pre-absorbed against rabbit and several other species, and the donkey anti-rabbit pre-absorbed against goat and several other species, which should guard against cross-reactivity. I also remember reading that the DAB reaction product (I believe it was used with the ABC system) would block binding sites, but could not find the reference yesterday so deleted this from my message. If you have the reference, I would be curious to see it. Tough to plug our products here because of cross-reactivity: as a general question, how often is this an issue in immunogold experiments? Rick Powell rpowell@nanoprobes.com Wed Apr 25

Triple labeling experiments can be done of course, but as was illustrated by Hong and commented by Powell, there are risks and pitfalls, especially when working with secondary antibodies. It is certainly not for the fainthearted and one would have to be lucky to get it right without some (and potentially a lot of) fiddling. The amount of controls involved can be mind-boggling. Blocking with a relatively high concentration of secondary antibody, tagged or not, brings its own risk of increased background and leaves a chance of cross-labeling, I do not think it can ever be absolute. If going with secondary antibodies I would choose a different approach: perform two or three double labeling experiments instead as follows: * antigen 1 and 2 * antigen 2 and 3 * antigen 1 and 3. Such an approach would in my view be imperative as a preparative step for validation of a triple labeling. The suggestions that were given for preventing cross labeling of antigens by blocking out epitopes on primary and/or secondary (DAB polymer, silver or gold enhancement) are more easily tested in a two-by-two set up. Mutatis mutandis, first of all the single labeling has to be established to work well for each individual antigen. The protein A approach for multiple labeling worked out in the 80's and 90's by Hans Geuze's group in Utrecht is a neat way to overcome the issues caused by a secondary antibody approach when having primaries of the same species. An intermediate step of free protein A (200 µg/ml) after the smaller particle size protein A gold conjugate incubation will efficiently block the first primaries. Free binding sites on either protein A-gold or the blocking free protein A will of course bind the second primary from the same species, but since an antibody has only one binding site for a protein A molecule, this is not a problem. This should work in triple labeling, even with three primaries from one species and if my memory doesn't fail me, the Geuze group has demonstrated this as well. Sorry Lee, it IS long and tedious no matter how you approach this. Jan Leunissen leunissen@aurion.nl Thu Apr 26

I agree with Jan, I tried the approach detailed by Rick some 15 years ago for immunofluorescence and it didn't give satisfactory results. It is very tricky to go around all cross-reactions. As Jan points out, making all the controls necessary may be very tedious and we shouldn't forget that we are taking immuno-gold in TEM here, which is much more time-consuming than immunofluorescence. I suggested direct labeling of the primaries but it has its drawbacks too. Stephane Nizets nizets2@yahoo.com Thu Apr 26

Instrumentation:
parts for older instruments

I need help from someone familiar with the DIC (Nomarski) equipment used with the Nikon Microphot series of microscopes. I am interested in transmitted light DIC with 160 mm objectives, not epi-illumination DIC. My understanding is that you need the Universal condenser, a DIC slider, and either a dedicated DIC intermediate nosepiece mount, or the EPI-FL3 unit with the 1.25× intermediate nosepiece mount, which has a slot for the slider. I purchased a condenser and Nikon slider (#59234) that was supposed to be for the Microphot. When used with my EPI-FL3 unit, the slider has the proper width and thickness to fit into the intermediate nosepiece mount, but it is much too long. It runs into the nosepiece before even the blank hole in the slider is in proper position. The slider measures 125 × 38 × 15 mm. I think it would need to be about 40mm shorter to operate properly with the EPI-FL3 and the standard nosepieces I have. What are the dimensions of the proper slider that goes with the EPI-FL3? Is there a special nosepiece that will allow my slider to operate with the EPI-FL3? What configuration is my slider intended for? I would also be interested in hearing from anyone interested in selling the proper DIC components to operate with my scopes (Microphot FXA and SA). ***Ralph Common common@msu.edu Mon Apr 2***

I'm not familiar with the Microphot in particular, but I can tell you that vendor support for older microscopes, especially fixed tube length systems, is pretty limited. It's a bit like vintage camera equipment—you can get beautiful images from it, but replacing a broken part or upgrading means being able to obtain vintage equipment from sources such as eBay, and generally being plugged in to a specialized group of legacy equipment users and hobbyists who will have the information you need about your older system. You didn't make clear who you got the slider from. Was it an eBay sale or from Nikon itself? In any event, I recommend trying to find Nikon literature from that era so that you can have all the part numbers for the Microphot compatible components you might need. Science-Info.net is a good resource. Here is it's list of older Nikon manuals, albeit, I don't see any specifically for the Microphot: http://www.science-info.net/docs/Nikon/ There's a dealer going by the name Classic Optics that might be able to help you with literature and possibly even the part itself: http://www.science-info.net/docs/Nikon/ I also *highly* recommend the Microscope Yahoo Group, which is a fairly high-traffic list with many members who are highly knowledgeable about older microscopes: http://tech.dir.groups.yahoo.com/group/Microscope/ Peter G. Werner germpore@sonic.net Tue Apr 3

TEM:
digital images with inverted contrast

Regarding images were taken at magnifications of 3,000× and below at 80 kV. The electron-dense regions appear bright and the electron-transparent regions appear dark. The CCD gain reference images are up to date, although they were not taken at the same magnification. No such problems were observed at higher magnifications. Has anybody encountered the same problem? Any suggestions on how to correct it? ***Hongwen Zhou hongwen.zhou@temple.edu Thu Mar 1***

The first guess is that you over-exposed the image. If your data is a signed 16 bit integer, going above 32K counts looks (to the computer) like a negative number. If you don't spread your beam as you drop magnifications or if there is a change in the lens states, you

https://doi.org/10.1017/S1551929512000466 Published online by Cambridge University Press

could easily get your brightness too high. This is an easy thing to test. Spread your beam with the C2 lens (intensity) or increase your spot number (C1 lens) to make the image less bright on the screen. At some point, the image should appear with normal contrast. Henk Colijn colijn.1@osu.edu Thu Mar 1

Can you see the image on TEM main screen at low magnifications (the same as used for CCD camera recording) in proper contrast? If not, I would suspect a misaligned LM mode of your TEM. In this case you could see a dark-field image (reverse contrast) of your sample. Oldrich Benada benada@biomed.cas.cz Mon Mar 5

TEM:

300 vs. 100 kV

I was wondering what is the reason for the low contrast of images in a 300 kV microscope compared to a 100 kV microscope. Technically the only difference is that you use a beam of 0.00370 nm for 100 kV and 0.00197 nm for 300 kV. Theoretically, if wavelength changes the resolution increases. It is unclear to me how this affects contrast. There is a small indication however that this affects mean free path by changing the probability of scattering (p) defined as thickness/ wavelength or something like that. Is it possible to have your 300 kV gun in F30 FEI microscope run in 2 modes 300 kV and 100 kV? Does anybody have a microscope configured and using it in this way? ***Regan reganhll@gmail.com Sun Mar 4***

There are two aspects to your question that might be worth separating: First, is there a fundamental physical difference between a 100 kV electron passing through an atom relative to a 300 kV electron? Second, does the electron optics of the microscope make a difference to the contrast at these two voltages? To the first question, the answer is yes, there is a two-fold difference. The electromagnetic potential of an atom increases the energy of an electron passing through the atom so that it moves slightly quicker than electron wave passing out in the vacuum. When it emerges from the atomic potential (slowing back to its original velocity if the interaction is elastic) the wave is phase shifted. This phase shift is bigger for a low beam energy electron (100 keV) than it is for a high energy electron (300 keV). The stronger phase shift, the bigger the contrast attainable if phase contrast imaging modes are used. Further, the chances of the beam electron scattering inelastically also rises as the beam energy drops. Specifically, the ionization cross section (probability of ionizing an atom) rises as the beam energy drops, so that contrast from inelastically scattered electrons rises too. Therefore, an 'average' sample looks darker, with more contrast at 100 kV than it does at 300 kV. Ray Egerton's book "Electron Energy Loss Spectroscopy in the Electron Microscope" examines the difference between elastic and inelastic in chapter 3 (I think). I've found this book extremely useful in the past and Ray's writing style is clear and accessible. I have no financial interest in recommending his book! The answer to the second question (electron-optical differences) is probably more subtle. Lower energy electrons are more easily deflected, so that 100 keV electrons are travelling at slightly higher angles relative to the optic axis. Aberrations in the lenses (spherical aberration and chromatic aberration) become more significant, resulting in, for example, lower point resolution (Cs lambda^3)$^{0.25}$ and larger chromatic blurring. The lower energy electrons striking your detector (negative/ CCD camera) will have a smaller interaction volume and will create fewer excitations. A fresh gain reference will be needed at the lower energy plus a fresh set of magnification calibrations. In answer to your question about configuring the F30 for 100 kV, the answer is yes you can. The Schottky gun emits electrons with a kinetic energy of about 4–5 keV into the HT accelerator which can be brought down to as low as 20 kV. For example, we have a FEI F20 that was aligned for 200 and 100 kV in the factory, but we have since aligned at 80 kV and 40 kV without difficulty. The only issue we have is the Gatan Imaging Filter (GIF) which we use to image our TEM images—the GIF usually requires a Gatan engineer to align it at a set energy ($$!). Fortunately, we were using it in STEM mode at 80 & 40 kV and detecting the electrons wasn't a problem with the BF/ADF/HAADF detectors. Jon Hermes jsb43@hermes.cam.ac.uk Sun Mar 4

There are two types of contrast that are of concern to biologists, so I'll restrict my discussion to those. For stained, conventional plastic sections, the mechanism of contrast is that some electrons are scattered to large angles primarily by the high-Z atoms of the stain. These electrons are intercepted by the objective aperture (if no aperture is inserted, some electrons hit the objective lens lower pole piece, some are back-scattered, etc.), and these electrons are, therefore, removed from the image, creating dark regions. For a sufficiently thin specimen, fewer than half the electrons are scattered—check the pixel values of the dark vs. light regions of the image on a CCD camera—so the difference in contrast between 100 kV and 300 kV is due to the difference in scattering cross-section, with 100 kV electrons being scattered about twice as often as 300 kV electrons. For thicker or more heavily stained sections, this is not as much a factor, since even at 300 kV enough electrons are scattered to give contrast, and scattering from the unstained parts of the section at 100 kV decreases contrast by increasing the background. The second type of contrast occurs for unstained specimens such as frozen-hydrated ones. This is phase contrast, which is caused by the difference in phase change undergone by electrons traveling through a slab of ice compared to those that have traveled both through ice and biological material. The phase change is caused by the electrostatic potential within the specimen. This will decrease the wavelength of an electron traveling through a positive potential and increase the wavelength of an electron traveling through a negative potential. The relevant equations are E = T + V, or total energy (which is conserved) is equal to kinetic energy plus potential energy, V = qP, or potential energy equals the charge times the electrostatic potential, and v/ lambda = ph, or the speed divided by the wavelength, which equals the circular frequency, is equal to the momentum divided by Plank's constant. (I think I got my factors of 2pi correct, but someone else from the list can correct me if I did not.). The changes in wavelength mean that the phase of each electron emerging from the specimen will depend on the potential it experiences along its path through the specimen. These differences are larger compared to 100 kV than to 300 kV, so for thin specimens, also referred to as weak phase objects, the contrast will be higher at 100 kV, all other things being equal. The principle other thing is the coherence of the beam. This has more of an effect on phase contrast than does accelerating voltage, and FEG sources are more coherent than LaB_6 sources operated in "tip mode," which are, in turn, more coherent than LaB_6 sources operated in the normal way with emission of electrons from both the tip and the pyramidal faces of the filament, or, least coherent, tungsten filaments. Imperfections in lenses, signal-to-noise ratio in biological images, and other factors such as the difference between the stain atoms and the biological features of interest, all have more influence on resolution than the theoretical maximum achievable due to wavelength. I hope this gives you a good introduction to contrast. There are any number of EM books that have several chapters each on contrast mechanisms and image formation. I suggest you find the one that most closely matches your knowledge of physics—which should not be completely lacking in a biologist, especially one who wants to see the equations. Finally, most scopes can be operated at several values of accelerating

https://doi.org/10.1017/S1551929512000466 Published online by Cambridge University Press

voltage. All that is necessary is to have good alignment files stored at the voltages of interest. (Older, non-computerized scopes require realignment every time the HT is changed, but I assume you do not have one of those.) Every 300 kV instrument I have worked with has at least factory and installation alignments at 100 kV, so one can choose the operating voltage pretty freely. Bill Tivol wtivol@sbcglobal.net Sun Mar 4

TEM:
high background contrast

We are using a JEOL JEM 2100F. But the contrast or diffraction from the amorphous carbon on grid is too high. Because of that, we cannot sometimes see contrast from our samples clearly. The same samples, when examined in a Technai F-30, give lot better contrast with background suppressed to almost invisibility! What can be the reason? ***Amit amit.welcomes.u@gmail.com Thu Mar 8***

This is interesting; I can think of a few things that may be going on. One is that the 2100F is a 200 kV field emission microscope and the F-30 is a 300 kV machine and is probably FE as well, so there is a voltage difference and the higher voltage machine will show less contrast from the carbon film—but it is not a huge effect. Another thing that I wonder about is carbon "contamination" that can wash-out contrast; I'm wondering whether you have a cold trap with liquid nitrogen operating—to reduce contamination in one machine over the other? Are you seeing contamination anyway? Are you using the same aperture angles? If you have a large objective aperture you will get less contrast from the carbon film—comparing aperture angles between microscopes is easy if you use aluminum or gold diffraction standards (used so you can check your camera length). Have you tried STEM? Robert Keyse rok210@lehigh.edu Thu Mar 8

You didn't say if you see the contrast on the negatives or on a digital camera. If you are using a digital camera, please check your reference images. If the reference images are not correct, they will add to the background noise instead of suppressing it. Mike Bode mike.bode@resaltatech.com Thu Mar 8

If your 2100F has STEM, try dark field for imaging small particles—they will jump out at you that way. In TEM mode check the auto contrast limits on the digital images, use a small objective aperture and try lower KV. What CCD camera and software are you using? If it is Gatan DM, change the gamma setting on the image to enhance the particle contrast. Roseann Csencsits rcsencsits@lbl.gov Thu Mar 8

TEM:
magnetic material

We work with different kinds of users that want to do TEM. Some of them want to analyze magnetic material—Magnetic particles in bulk. Last week we had an accident with a sample. The sample was some kind of iron alloy prepared by dimpler polishing. A large chunk of if broke inside the objective lens (OL) of our TEM-FEG. Very fortunately, that piece came out by itself. No need to call the service engineer to disassemble the OL and remove the broken sample. I was wondering how other EM labs deal with this kind of material. Do you have some kind of special protocol or just don't accept this kind of sample inside the TEM? We have a service contract for the microscopes, but I heard from the service engineer that using a magnetic material in a TEM is a misuse and damage arising is not covered by our service contract. Some of my colleagues argue that we can load any kind of sample in the TEM, and this should be covered by the contract. Any comments? What about magnetic particles? We have several users that study nanoparticles. Magnetic or doped by magnetic elements like Co. Do you think these particles can fly off from the grid and get on the OL? If these contaminate the OL, can it affect the performance of the TEM? ***Carlos Kazuo Inoki carlos.inoki@lnls.br Sun Mar 4***

It's tough working with magnetic samples these days, as the instruments do not have systems that help the operator. With the development of magnetic materials in the late 1960s the TEM exchange system allowed the specimen to be lowered into the objective lens from above, this enabled workers to manipulate the objective focal length, hence lower the level of magnetic field. This was very easy to perform and quite good magnetic domain work was achieved. Some manufacturers even had attachments for this work known as Lorenz (? Spelling) accessories. Today I suggest using the Z prime or eucentric setting to lift the specimen as far away from the lower pole piece as possible (focus will move anticlockwise) to lower the objective lens strength. Another area of gain is to combine this with a lower accelerating voltage. Be aware the magnification will be lower than indicated due to the lower objective lens current. Try it and see the benefits? Steve Chapman protrain@emcourses.com Mon Mar 5

I did my Ph.D. thesis analyzing steel samples, all highly ferromagnetic. The only time I had a problem was when the field of the lens pulled on the sample hard enough to overcome the snap ring and pulled the entire sample out of the holder. Well, that was a 100CX JEOL, and it was easy enough to disassemble and reassemble myself, and there was my sample hanging from the pole piece, all in one piece. Newer microscopes can be a lot more complex. I worked for 1.5 years at Komag division of Western Digital doing TEM of magnetic media. The total amount of magnetic material in those specimens was actually quite small and we never had any problems. I was told by the SEM folks, though, that over time, small grains can separate from full computer disks, especially if they were cut or scratched, or damaged during testing, and that these small particles would accumulate in the SEM final lens pole piece such that cleaning was required every few years. Apparently the biggest problem was in SEMs operated in immersion or snorkel mode. John Mardinly john.mardinly@asu.edu Tue Mar 6

TEM:
difference between "shift" and "bright tilt"

While using a JEOL 2100F TEM, I noticed that the "Bright Tilt" button on left panel alters one of the deflector coils above condenser lens (CLA1) while the beam "shift" knob alters the other deflector coils (CLA2). Why is one labeled "Shift" and other one "Tilt"? Is there any logical explanation on when to use which one for bringing beam to center? ***Amit amit.welcomes.u@gmail.com Mon Apr 16***

Both the gun and the complete illuminating system, gun plus condenser lenses, need to be centered and tilted to place the electron beam absolutely straight down the magnetic axis of the system. In the case of the gun, it is "shifted" onto the screen center and the angle of the beam in relation to the magnetic axis of the first condenser lens is adjusted by the gun "tilt" controls. In order to make this alignment the second condenser is brought to crossover, imaging the virtual source. This action is best carried out at a magnification that makes the image about 4 cm across. The gun shift must be used to center the beam on the screen whilst the gun tilt is used to evenly balance the halo of the desaturated source. In the case of the illuminating system, once the gun is aligned as described the complete illuminating system is shifted to center the beam on the screen and tilted to place the beam on the magnetic axis of the objective lens. The latter action depends upon the microscope. Japanese instruments allow this alignment to be carried out either to the current centre of the

objective lens or to the voltage center. European instruments simply align the current center. For the current alignment the objective lens current is changed and the illumination tilt used to center the spinning pulse on the screen. In the voltage alignment case, the high voltage level is changed and the illumination tilt is used to center the image pulse on the screen. The changing the illumination tilt controls will change the center of the magnification/diffraction pattern on the screen thus further "projector" alignment may be required. When aligning the instrument it is very important when working on the gun to only align the instrument with the gun controls; a big error made by many! Under normal operating conditions the illumination should be kept in alignment with the illumination shift controls. This action is essential because the shift has no influence on the voltage or current alignments, which are very critical alignments. Steve Chapman protrain@emcourses.com Mon Apr 16

SEM:
immersion lens imaging of nanotubes

Are there any issues imaging carbon nanotubes in an SEM under an immersion lens setting? ***Chris Meyer cmeyer911@yahoo.com Thu Mar 15***

I've done this. No issues other than the usual getting-the-best-image issues. Philip Oshel oshel1pe@cmich.edu Fri Mar 16

We never had problems with nanotubes using with in-lens for imaging. Just once, our user was analyzing some kind of loose fiber and a small fiber got inside the lens and start causing problems with the imaging. But this fiber was enormous in size if compared with nanotubes. After that we start to ask our users to use an air blower to remove any loose piece of sample on the carbon tape. Carlos Kazuo Inoki carlos.inoki@lnls.br Fri Mar 16

I observed much NTs grown on different kind of substrates and had no problems. But a colleague observed other types, with Ni or Fe as catalyst and where the NTs are free to move, and we must regularly clean the OL bore from the dust, which is trapped by the magnetic field. Since then, I require users to pass the sample holder under a strong magnet (from a old hard drive), to catch all what could be free to fly away. It's a little bit better, but is not perfect. Jacques Faerber jacques.faerber@ipcms.u-strasbg.fr Mon Mar 19

SEM:
membrane filters

We need to purchase membrane filters for blood and other cells examination with an SEM. As I see in the market, track-etched filters have single pores that make a much better background. There are two types available: polycarbonate (PCTE) and polyester (PETE) track-etched membranes with identical shapes. PETE is more resistant to solvents than PCTE. If anybody has used both, I would like to ask if there is any difference in SEM imaging due to possible differences in electron conductivity or other properties that may affect the quality of imaging. ***Yorgos Nikas eikonika@otenet.gr Fri Mar 9***

We have been using polycarbonate filters, 12 mm diameter, 0.2 micron for the preparation of just about all kinds of cells. The results were always great. Recently we started seeing crystals on the filters in the SEM. After much trial and error, we read the notice on the manufacturer's packaging stating that a change has been made to the product. Apparently some kind of coating is now put on the filter and this, we think, crystallizes with dehydration or critical point drying. Anyone with a similar experience? Alan Hall Alan.Hall@up.ac.za Fri Mar 9

No, fortunately not, so far. But from my point of view, it makes sense to confront the manufacturer with your results (send them an example SEM image before and after change of the product!) and ask for a statement, or improvement of the product, the filter. Reinhard Rachel reinhard.rachel@biologie.uni-regensburg.de Fri Mar 9

SEM:
asphalt

A researcher wants to examine solid asphalt specimens under our high-vacuum SEM. Is it safe to the SEM. Would that contaminate the column or the TMP? ***Ahmad Ashkaibi ahmad_ashkaibi@hotmail.com Tue Mar 27***

Your question is rather timely. I had someone asking to look at some samples of concrete yesterday. Like them, I wonder if your researcher has thought things through very well. What do they want to see? How do they expect to do so? What kind of preparation and magnification will be required? Those issues may pose bigger issues. Our first attempt to look at concrete in an SEM goes back almost 20 years. We prepared a piece between 1 and 2 inches square and about 1/4 inch thick, coated it with gold, and placed it into the SEM and started pumping. Our JEOL 840A gave up after some time (30 minutes?) as it was not able to move beyond the roughing cycle. We were able to bypass the timer and left the SEM pumping over night. It had reached working vacuum by the next morning. However, examination showed extensive cracking where the vacuum had pulled water out of the structure. (I don't recall how completely cured it was.) Therefore, high-vacuum characterization was not an option. We purchased an SEM with variable pressure capabilities and have used it for over 15 years. Most of our work is done at 40 Pa (~0.3 Torr). That means we achieve operating vacuum quickly, the residual atmosphere alleviates charging, and samples are not grossly changed by exposure to high vacuum. You definitely have a non-conductive sample that either needs coating or needs to be examined in V-P mode. I don't know that asphalt samples would have such troubles with out-gassing, but I expect they would. A sample might seem stable at room temperature and pressure, but the lower pressure of an SEM would extract additional volatile components. That would change the nature of your sample and present pumping and possible contamination issues. The binder would heat some under the beam and that would release more components. As the binder loses components, I would expect any conductive metal layer to be disrupted and the sample would start charging. A cold finger might help capture those components so they don't condense somewhere bad. Your cold finger would then need cleaning. One significant benefit of V-P mode is that the residual gas changes the pumping regime. It helps to sweep contaminants out of the chamber rather than letting them diffuse throughout and condense on surfaces. For those reasons, I would not try to examine the sample in high-vacuum. I might let them try to examine a small piece, but I expect the examination would be a failure because of changes to the sample. I would try V-P mode, but I would watch for changes in the sample to see if you are truly seeing the sample as it is. That brings me back to the questions of what they want to see and how they will prepare the sample. If they simply grab a blob of asphalt concrete, they will see the aggregate shapes and should be able to see the gross structure, including large-scale porosity. However, if they zoom in on the aggregate, they will see most, if not all, of the aggregate coated with binder. The beam will have difficulty penetrating through the binder to the aggregate. A stereo microscope could do about as well. If they try to prepare a section through the sample, they will be able to discern things at low magnification. I would be concerned about the binder migrating over the surface and causing difficulties at high magnification. Again, a stereo microscope or reflected light microscope might do as well. So I would ask again, why are they considering SEM, what do they hope to learn or document, and how? I encounter many students who really do not consider those questions

before bringing me their samples. Help them to bring those issues into focus. Warren Straszheim wesaia@iastate.edu Wed Mar 28

Unless you have a cold stage and use a very small sample on that stage, you are probably going to contaminate your system. Is that a problem? It depends on how the system has been used in the past and how you intend to use it in the future. If you are running LaB_6 or it has a field emission gun I would not put the asphalt in the system without a cold stage. On the other hand, if it is an old tungsten gun and the TMP is brought to atmosphere with every specimen change, you may not notice any additional contamination. You can also lessen the amount of contamination by operating at a low kV and small spot size (high condenser lens current) to minimize heating. If you need to do X-ray analysis, you are going to heat the sample. Period. If the system has been kept very clean and quantitative analysis is a major part of its job, I would not put asphalt in. On the other hand, if it is an old system and no one has been particularly careful about what goes in, and it's mostly used for imaging and maybe some qualitative analysis, then I might consider it. To actually answer your question, yes, it is going to contaminate your vacuum system unless you have a very small sample on a cold stage. If the system is currently very clean, then yes, it would be considered damaging to the system. Ken Converse kenconverse@qualityimages.biz Wed Mar 28

I agree with all your statements but one: "SEM with variable pressure capabilities . . . at 40 Pa (~0.3 Torr). That means . . . samples are not grossly changed by exposure to high vacuum." Pressure 0.3 Torr is just 0.4% of atmospheric pressure. It means that compared to pressure at high vacuum mode 99.6% of pressure difference is still applied to the specimens. For my porous specimens (bone, teeth) I do not see any difference in crack development at high vacuum mode or at variable pressure. The same goes for water: only 0.4% of partial water pressure could be preserved at 0.3 Torr, so water would evaporate rather quickly, at about the same rate as in high vacuum mode. I believe specimen protection from difference in pressures and/or any water preservation in specimen are not among benefits of variable pressure mode. Vladimir M. Dusevich dusevichv@umkc.edu Wed Mar 28

SEM:
image distortions

We are experiencing distortions/interferences (please check an example here: www.lucafedele.eu/test1BSE5500.jpg) when capturing images on our Camscan Series 2 through our Gatan Digiscan II interface. The images look ok on the old green phosphorous screens (but it might be because their resolution is limited, of course) but come out distorted when captured. I have attached a BSE sample at high magnification (5000×) but the effect is also visible with SE images and at lower magnification (1000× or so). The effect remains regardless of software settings. We have several cables running around the SEM and suspect some sort of electrical noise (which could also be generated inside the computer connected via Firewire to the Digiscan) but so far, we have not been able to find a cure. If anybody has ever seen "interference" like this one and has any kind of suggestion, we will be more than happy to give it a try. ***Luca Fedele lfedele@vt.edu Mon Apr 9***

I am working on a SEM and found that some of the spikes (line distortions) might be generated by a mobile phone nearby. I would also check for mechanical vibrations first (do you have a turbo-molecular pump?) and then check all earth connections. Stefan Diller stefan.diller@t-online.de Mon Apr 9

It really looks like mechanical vibration to me. I have seen this kind of noise in images when the scope is being subjected to mechanical vibrations, but also when the sample is not well clamped down inside the chamber the beam can cause sample movement too resulting in vibrations along the edges of objects in the image. Although, this usually requires much higher magnifications to see. Nick Seaton seato008@umn.edu Mon Apr 9

You say that is a BSE image. The brightness variation is a bit much for a BSE image. Since I see you are with the Department of Geosciences, I suspect charging is present. It may be contributing to the appearance as much as any electrical interference. I would suggest two things. First, I would recommend cutting back on current if it is higher than normal. If you are truly doing everything else (coating, grounding the sample. voltage, etc) the same, then high current may be leading to charging. I presume you have EDS on this system. What kind of total count rate do you get under these conditions? Second, I would just put this sample aside and examine something conductive like a block of metal or a sample holder. If it shows distortion, then you know you have some other problem to track down. Warren Straszheim wesaia@iastate.edu Mon Apr 9

Just another idea: Does your SEM have some filters in the signal-path which change bandwidth with the configuration of line/frame settings? It might also be a wrong filter bandwidth contributing to this kind of signal if your image acquisition system does not have its own scan generator. Stefan Diller stefan.diller@t-online.de Mon Apr 9

To judge exactly what is happening, you need to help us by providing more information; one picture does not tell the full story! Use the standard fault finding procedure—"Is the 'fault' the same at short and long working distances?" If so it is almost certainly a vibration, if not then it is almost certainly a magnetic field. Vibration should not change no matter what the working distance or accelerating voltage. Magnetic fields are reduced at higher accelerating voltage or at very short working distances the specimen being protected by the higher lens fields. Steve Chapman protrain@emcourses.com Tue Apr 10

Checking the effect of accelerating voltage is a good idea to ensure magnetic field or not. One way to check vibration is to design an assembly to fix a sample to the lens (as a backscattered detector can be) and not to the stage. Like that, if there is vibration, sample and beam are synchronized and you should not see effect on the image. I would be very interesting to check carefully the green screen to know if the aberration is same or not (by increasing magnification?). Nicolas Stephant nicolas.stephant@univ-nantes.fr Tue Apr 10

The fuzzy edges along the grain boundary are likely caused by mechanical vibration interferences. You would see this effect with any other detectors if the vibration source remains. The problem will be resolved once the vibration source is spotted and removed. Make sure no external object (e.g., table) is in direct contact with SEM main console. Cables need to lie loosely on the ground. It is also a good idea to put a rubber mat under the rotary pump. Xiang Yang xyang@smu.ca Tue Apr 10

The interference that you have in your image looks pretty periodic. Have you tried to measure the periodicity? The periodicity is often a good point to start. If it is 50 or 60 Hz, it is most likely an electrical problem, which could be due to ground loops. In that case, I would suggest connecting the entire acquisition system to the same power source and ground as the microscope. I have also seen this when the start of the line acquisition is "at random" with respect to the phase of the power signal. You may want to synchronize the start of the line acquisition with the phase of the power signal. If the periodicity is not 50 or 60 Hz, I would check out the sources for noise already mentioned here. You may also want to check out any electrical equipment that is nearby. A colleague of mine found out

after some sleuthing that the spurious signal he had in his data came from an elevator quite a distance away. They ended up blocking the elevator when they had to make sensitive measurements. Mike Bode mike.bode@resaltatech.com Tue Apr 10

Core Facility:
biohazardous samples

A scientist has asked to image porcine virus in our FESEM. She says that it is listed as a biosafety level 2, but has been disinfected to >99% level with 10% glutaraldehyde. Are any of you working with this virus? I'm not familiar with the safety protocols. Is this a safe project for a core facility? ***Owen Mills opmills@mtu.edu Fri Mar 30***

It isn't really up to you or the principal investigator to decide if this is safe to do. Your university undoubtedly has a biosafety committee. Only they can approve the use of a BSL-2 pathogen in your core facility. The principal investigator needs to have a protocol in place that has been approved by the institutional committee. Tom Phillips phillipst@missouri.edu Fri Mar 30

Unless it is true and verified (whatever one means when saying "fixed") that the virus (suspension, particles, precipitate or whatsoever) previously has been "classically" fixed (conformal change of protein structures e.g. by suitable aldehydes) [deactivated by "10% glutaraldehyde-solution" I don't think the virus particles to be infectious any more so these don't need to be included under BSL-2 pathogen strategy. There are some papers concerning deactivations for personal as well as working safety by e.g. 1–2% formaldehyde and/or 1–2% glutaraldehyde, so the question rather is whether you'll see or can image something of "fine" structure of virus particles after 10% glutaraldehyde (only diluted?, buffered?, incubation time for deactivation?). The ultrastructural aspect of virus particles deactivated that way has been reported to be of poorer quality than images taken after classical negative staining. We had recently an extensive discussion on the listserver about the preparation of HIV particles for TEM. The actual question is not whether or not the virus is inactivated and still dangerous after fixation. The real issue is regulations. BSL-2 implicates that specific measures are undertaken not only to protect the person who manipulates the samples, but also all his colleagues who are perhaps not even aware that someone is working with a BSL-2 virus next bench/door. Please read carefully the regulations about BSL-2 and, as already advised, discuss with the right persons about it. I mean, not electron microscopists but security/health managers. There are strict regulations and protocols which must be strictly followed; it is not as if anyone can do anything with BSL samples. Stephane Nizets nizets2@yahoo.com Tue Apr 3

Core Facility:
fees

I have a question concerning the TEM user fee charge rate for internal and external academic users. I noticed the common practice is that the internal academic users are charged at a lower rate than the external academic user. This sounds reasonable. However, I am told by my administrative people that we are not allowed to charge federally funded users rates that include unallowable costs. Whatever it means, we are now charging the same rate, which does not sound right to me. I would appreciate if anyone can give me some information/rationale on the different academic rate. ***Yan Xin xin@magnet.fsu.edu Mon Mar 26***

When I was working for New York State, we were by law not allowed either to make a profit or to lose money from outside users, so someone above my pay grade had to calculate the exact hourly cost of running the facility. I don't know whether this is the case in Florida. In any event, all outside users had to pay the same amount; however, since we were an NIH Regional Resource, users with NIH grants were not charged anything. Sometimes the charging structures can be pretty complicated and not altogether rational. Bill Tivol wtivol@sbcglobal.net Mon Mar 26

Setting fair rates is indeed difficult for a university core facility. Different institutions have slightly different policies but a few things need to be considered: (1) The federal granting agencies do require that the rates are the same for all those who have federal grants and that these are equal to those paid by others at the institution. This means that you cannot allow some users to pay less than others. Sometimes there is a desire to give new users a break or arrange other incentives but this is not acceptable if it means that others with NIH or other grants pay more. (2) External non-profit users are normally charged the same base rates as internal users with the following exceptions: They normally must pay the indirect charges that each institution also deducts from grants received by internal researchers. These funds go to maintain the institution facilities and pay for heat, electricity, etc needed to run the facilities. Often labor is subsidized for internal users in one way or another. External users usually have to pay full salary + fringe benefit costs for the labor associated with their using the institution facilities. (3) External for-profit users pay all these costs plus additional. Since the university is not-for-profit, they must not charge less than commercial sources for similar activities. Thus rates for external for-profit users are normally significantly higher than those for internal or external not-for-profit. You need to get an idea of external rates from other institutions and commercial labs in your area so that you can justify your rates. Debby Sherman dsherman@purdue.edu Mon Mar 26

If I understand you correctly, your administrator has directed you to charge both external and internal users the same fees. Unless there has been a change in Federal policy, the only injunction applies to fees—for the use of instruments purchased/supported(?) with Federal funds—that unfairly complete with private businesses in the local region. Where there is no such competition, there can be no issue. I have always counseled—to those who care—that there are some applications of microscopy in which I am not interested such as quality control. Thus, I have been willing to train operators from a private external corporation who can then pay an expanded hourly rate that does not unfairly compete with similar charges of private suppliers of microscopy services. If I managed a facility at the University of Chicago and wanted to open the facility to 'work' sent from private corporations for microscopy services I would have the following considerations (admittedly somewhat complicated) to prevent me from unfairly competing with McCrone Associates capabilities (http://www.mccroneassociates.com/techniques). 1. I could charge fees exactly like McCrone's for similar services. 2. I could offer to McCrone, as to any other organization, access to those instrumentalities that are not included in the McCrone possessions that I happen to have in my facility. I could refer to McCrone as McCrone could refer to me—very collegial! 3. I can charge internal users whatever the financial administrators of the University of Chicago felt would be accepted by the next funding agency reviewers who will fund charges for use by "fundees," but will generally not fund requests for lump sum service contracts. This work is for institutional bean counters and the cadre of users who work in the institutions and win the grant money administered by them. My point is that only Florida State, not Yan Xin, can adequately determine its potential for breaching the 'intentions' of the Federal regulations. Policies that I like to apply must be approved by superiors. Indeed, when I speak to superiors about these issues, I always try to bring a small stack of

papers that correctly illuminate the Federal Regulation and what I consider to be viable options for the facility I manage—which, by the way, has never been at an institution with large amounts of Federal funding. If you wish, you may contact me privately for copies of those documents that I carry with me to meetings with administrators who "run the joint." If you are going to manage, my policy is to go informed. My first fee schedule was derived from those I could take from facilities at institutions that had large amounts of Federal funding. My rationale was that such fees would generally reflect fees that would be acceptable to the Federal funding agencies like NIH and NSF. Frederick C. Monson fmonson@wcupa.edu Tue Mar 27

TEM:

LaB6 filament

Can anyone please share your experience of using the LaB6 filament on your TEM, particularly if you have an FEI Tecnai 12? What operating vacuum in real life is required so you don't blow the LaB6? Background: My old LaB6 (15 micron tip microflat) was purchased from Barry Scientific (apparently no longer in service). In spite of maintenance shut-off, building power shut-off, and vacuum crashes during specimen insertion, it lasted 5.5 years with an operating (gun) vacuum of 6–13(log scale, or <<10^{-7} torr). The newly replaced LaB6 (bought from Barry Scientific and stored since 2008) only lasted 4 months and blew not when the scope was busiest. BSc reassured me that their filaments can be stored for a long time. My Tecnai 12 TEM had its column opened twice for O rings replacement in the last several weeks. One more set of O rings to replace since vacuum is still sub-optimal. Vendor was convinced that a log value under 30 should be fine, and my scope was operating between 19–25. When the LaB6 blew, the operating vacuum was at 20. By the way, a log scale 22 is equivalent 0.35^{-6}. The Electron Microscopy Sciences document indicates that for LaB6, operation vacuum should be at 10^{-7} and work function (eV) should be ~2.70. My operating eV was ~2.30. Is operating at a vacuum between 20–30 pushing the limit? Any suggestion, even about handling, maintenance, or storage, would be appreciated. ***Fanny Chu fsmsc@hotmail.com Thu Mar 15***

To me (and I imagine to many other non-Tecnai people) the log reading is pretty meaningless. However, if a log scale of 22 equals 0.35^{-6} T, that would be 3.5^{-7} T which is OK. Not terrific, but OK. If you meant 3.5^{-6}, that is terrible and could explain the short filament life. Ken Converse kenconverse@qualityimages.biz Fri Mar 16

The mystery about my LaB6 was solved. It's embarrassing. I have just found out this moment that the filament that blew was a tungsten one, not a LaB6!! Somehow there was miscommunication, so a tungsten one was used. Many thanks to Ken Converse for his input. Fanny Chu fsmsc@hotmail.com Fri Mar 16

Fanny, we have two FEI CM20 TEMs that use LaB6 cathodes. We have used both Denka and Kimball Physics cathodes and get very long life (many years). The key is to be sure the vacuum on the IGP is good (we never turn up the cathode when IGP is >20). Even more important is to saturate slowly (I wrote a plug-in for DigitalMicrograph that turns it up in 10 sec increments) and to check the saturation after 15 min—the cathode continues to heat and can oversaturate. On our cryoscope, we have a turbo-pumped air lock and see no major jump in IGP on sample exchange, so I will change a sample with the cathode backed off a few clicks; the other scope only uses the mechanical pump and we get frequent vacuum jumps. I turn down the cathode and wait a bit before sample exchange. I'd rather take a few moments to have a sip of coffee while I wait than to have to spend the time changing the cathode and conditioning our gun to 200 kV . . . John Minter jrminter@rochester.rr.com Sat Mar 17

TEM:

lattice micrograph of river sediment fines

I have a micrograph (100 kV, 100 Kx) of what looks like lattice fringes, however the spacing is much too large (~10 nm between the lines). The "lattice" is very regular in spacing and perhaps is the result of clay layering, as the sample contains the fine fraction of river sediment including clays. Any ideas of what this may be? ***Jennifer Tully mille638@muohio.edu Fri Apr 13***

Look up Moiré fringes. This is an interference effect that occurs when two crystals are misoriented slightly and both are diffracting. The interference period is a function of both d-spacings and the misorientation angle between the lattice direction. Layered materials (clays) can easily do this. The spacing of the interference fringes can have very large "apparent" d-spacings. For a detailed discussion, see, for example: L. Reimer, *Transmission Electron Microscopy*, Chapter 8, pages 359–61 (1989). Vol 36 Springer Series in Optical Sciences. Nestor J. Zaluzec zaluzec@aaem.amc.anl.gov Fri Apr 13

EDS and XRF:

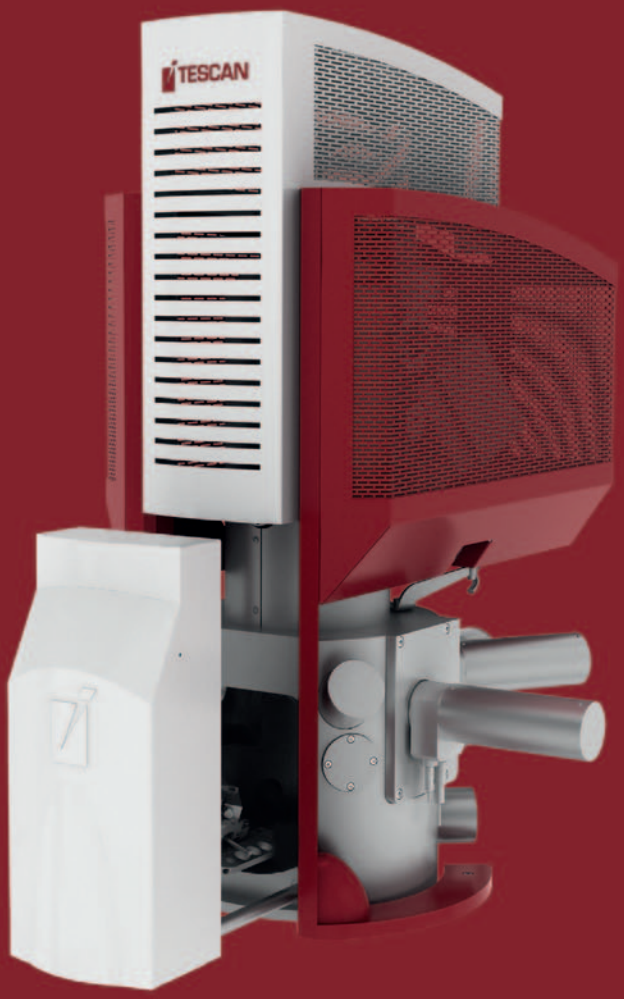
definition

I am a little bit lost with some definitions and would appreciate your help on this matter. The main question is: Is EDX (EDS, Energy dispersive spectroscopy) a part of XRF (X-ray fluorescence)? When I visit the definition of XRF in Wikipedia: http://en.wikipedia.org/wiki/X-ray_fluorescence, I can read that "X-ray fluorescence (XRF) is the emission of characteristic 'secondary' (or fluorescent) X rays from a material that has been excited by bombarding with high-energy X rays or gamma rays." Because in TEM/SEM, the X rays are produced by bombarding the specimen with electrons (and not X rays or gamma rays as in the definition), it seems that EDS in TEM/SEM is not a part of XRF. But later on the wiki page, I read that EDS is actually XRF. I would be grateful for any comment. ***Stephane Nizets nizets2@yahoo.com Thu Mar 8***

Wiki pages are not known for their technical accuracy; be careful when you use them. X-ray Energy Dispersive Spectroscopy (XEDS) is a methodology used to measure an X-ray emission from a material using technologies, which typically (but not exclusively) employ solid state detectors and then graphically display the result in the form of a pseudo-continuous spectrum. The detectors used provide a signal that is proportional to the energy of the radiation that is absorbed within their matrix. The signal is then amplified, processed, and displayed as a spectrum as a function of energy that created the signal in the detector. There are a number of different detector technologies that can be used and include: Si(Li), HPGe, SDD, HgI, Superconducting Transition Edge Microcalorimeters. XEDS has nothing to do with the excitation mechanism by which the X rays are actually generated. The X rays can be created by any process, incident photons, electrons, ions. So saying that "EDS is actually XRF" is a misnomer. XRF is the signal excitation process, that is, X-ray emission from a specimen created by fluorescence (that is, absorption of an X ray and the subsequent emission of X-ray photons of a different energy). This is different from the electron column case, which is an electron-in photon-out process. You can use any number of different detectors to measure an XRF signal. Of course, we are tacitly assuming that the article implies that XRF is being used to create X rays! X-ray absorption can also create numerous other signals, including electron emission; this process is called X-ray Photoelectron Spectroscopy (XPS)! Nestor J. Zaluzec zaluzec@aaem.amc.anl.gov Thu Mar 8

MT

Time for change?



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