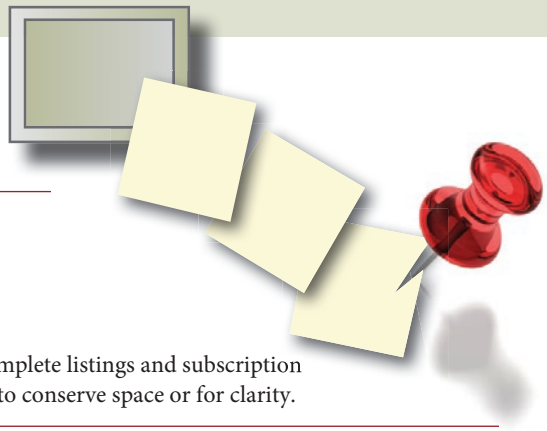

NetNotes

Edited by Thomas E. Phillips
University of Missouri

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Specimen Preparation:
SEM of eggs of sea bass and sole

We want to do SEM analysis on eggs of sea bass and sole. For sea bass, this is no problem, but when we apply the same protocol on sole eggs, the eggs shrink as soon as the dehydration protocol starts. This is our protocol: fixation in HEPES with 2% paraformaldehyde and 2.5% glutaraldehyde for several weeks, post-fixation in 1% OsO_4 for 2 hours. Then, we start the dehydration process in 50% alcohol up to 90%, finally acetone, followed by critical point drying (CPD) and platinum sputtering. I really hope that some of you can give me some advice. ***Wim Van den Broeck wim.vandenbroeck@ugent.be Thu Jul 4***

You could try something that works on watery plant cells: Fix in methanol. It works really well and gives the least shrinkage. Ref: Neinhuis C, Edelmann HG: Methanol as a rapid fixative for the investigation of plant surfaces by SEM. *Journal of Microscopy* 1996, 184(1):14–16 http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2818.1996.d01-110.x/abstract. Modified as follows: Leaf pieces were immersed in 100% dry methanol for 10 min, followed by 2 × 10 min changes in 100% dry methanol. Tissue was then critical point dried immediately with methanol as the transitional fluid. Alternatively, tissue can be transferred into fresh dry ethanol and left overnight at 4°C (essential for larger tissue pieces which retain water for longer). Tissue pieces were then critical point dried with ethanol. Essential is CPD straight away, no storage in solvent. Rosemary White rosemary.white@csiro.au Thu Jul 4

Specimen Preparation:
removal of gold coating from analytical samples

Some researchers at my university are searching for a way to remove a gold coating from analytical samples. The samples consist of Al discs containing indium plugs into which individual zircon grains have been pressed. I don't want to use iodine in KI because it reacts with the Al. Mechanical polishing is probably out because of the indium; don't ask about my attempts to polish indium! Does anyone know of any other possibilities I should look into. I seem to remember this discussed before, but I cannot find anything in the archive. Any help gratefully appreciated. ***Glenn Poirier gpoirier@mus-nature.ca Thu Jul 25***

If polishing or KCN etching are not compatible with the sample material, and if the sample is either sufficiently small or a small fraction can be broken off from it to fit into the ion mill, then I would try broad-beam Ar or Xe milling. Valery Ray vray@partbeamsystech.com Fri Jul 26

Or become friends with someone who owns a FIB.—Editor

Specimen Preparation:
plasma cleaning

May I know if you have any advice for cleaning the samples that are supported by the carbon films on the copper grids? Any comments on using H_2/O_2 gases as what is implemented in the Gatan Solarus? As I am using a Fishione Nanoclean, I am thinking that H_2 gas with O_2 gas will yield the same result. Due to the safety reasons in the lab, the safety officer suggested that I get a H_2 generator rather than H_2 gas cylinder, but it is quite an investment. I would like to thank Fishione, which also kindly provide me with some recipes on this cleaning protocol (but not based on H_2/O_2). On the other hand, though, I am hoping if someone could share some advices on this too? ***Tay Yee Yan one_twinklestar@yahoo.com.sg Sun Jul 21***

I have a couple of suggestions to consider. The first suggestion is simply to try the O_2-Ar mix. You may be surprised how well a carbon support film will hold up to an O_2 plasma. I was. Try a two minute run at first and work it up to a five minute run. The second suggestion is to simply try an Ar plasma. Replace the mixture gas that you are using with the Fischione unit with just pure Ar. The Ar by itself may give you what you need. The third suggestion is to try a mixture of H_2, O_2 and majority Ar. I would try about 10% O_2, 10% H_2, and balance Ar. This should not be dangerous. When a mixed gas plasma comes on, the more easily ionized gases will ionize first at lower power. As all of the gas is ionized, then the less ionizable gases will kick in. As you raise the power, the easy gases go first followed by the less easy. I'm guessing here because I don't want to look up the numbers, but I would guess that O_2 would be the easiest, followed by H_2, followed by Ar. Now in a plasma cleaning system, you typically will operate at low power and what gases get ionized will depend on their concentrations. You can easily order a lecture bottle of a gas mixture to try this out. Scott Walck s.walck@comcast.net Sun Jul 21

Specimen Preparation:
glow discharge

Is there any one that can simply explain theoretically what glow discharge is, how it works and the relationship between current and polarity? ***Robyn Leidel robyn.leidel@utsouthwestern.edu Tue Aug 27***

Glow discharge is the generation of plasma by an electric current. On a coater or other machine that uses a glow discharge or sputter process, polarity refers to which way the head is biased (positive or negative) relative to the sample (ground). When the plasma is generated, positive ions will be accelerated towards the negative end of the circuit and vice versa. So polarity controls whether your sample is bombarded by cations or anions. Current is the number of electrons flowing through the generated plasma to complete the circuit and can be considered proportional to the amount of plasma generated. In sputtering, the plasma is generated from a noble gas with the intention of bombarding a target with a process gas to free material from the target and coat a sample. In glow discharge for grid preparation, the plasma is made up from atmosphere (oxygen, nitrogen) which is very reactive. The end goal is to make the grid hydrophilic, which would mean adding more polar character (breaking C-C/C=C and C-H bonds to make various bonds with nitrogen and oxygen). Jacob Kabel jkabel@mail.ubc.ca Wed Aug 28

First sentence is problematic. Instead, glow discharge occurs when the generation of plasma is sustained in a chamber at right conditions of V (electric field), P, and species. Current is a result of glow discharge. Chaoying Ni cni@udel.edu Wed Aug 28

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In my knowledge, glow discharge is obtained with an AC 50/60 Hz voltage and not a DC. With an AC voltage, all the surfaces are bombarded by the ionized gas, and if the current is high enough, there is together sputtering and re-deposition everywhere. Works well for weak organic contaminations, but does not replace an RF plasma cleaner with Ar/O_2 or H_2/O_2 mixture. Jacques Faerber jacques.faerber@ipcms.u-strasbg.fr Thu Aug 29

Specimen Preparation:
Lowicryl K4M embedment

We have been having challenges with FluoroNanogold labeling of a very stubborn antigen on the surface of lipid droplets in murine brown adipose tissue. We use a rabbit polyclonal primary antibody and the goat anti-rabbit Fab' FNG probe. We also have now tried embedment in Lowicryl K4M with high-energy UV polymerization and using benzoin ethyl ether in the resin. We use dry ice chips for an O.N. polymerization to lower the temperature to try to preserve the antigenicity. Unfortunately, the chips evaporate by the time the next morning rolls around. The lamp distance is approximately 7 inches. The whole set up is tented in Al foil and the styrofoam box is also lined as well. Our first attempt of this at RT and UV polymerization failed to detect the antigen and the ultrastructure was not good. The only great thing was that the lipid droplets retained their slate grey tone, indicating that lipids were preserved. LR White tends to dissolve our lipids, but in other tissues, antigenicity is well preserved. ***Vickie Kimler vakimler@med.wayne.edu Mon Aug 26***

Do you have access to a -20°C walk-in freezer, or even the freezer compartment of a standard fridge? Similar to your system, we used a foil-lined cardboard box but with a "black light" bulb like those used to illuminate posters back in the '60s. We've used dry ice or liquid nitrogen to contribute an atmosphere allowing LRWhite and K4M to polymerize with good success. Also, molecular probes now distributes a secondary conjugate with a fluorophore and -10 nm gold that works exceptionally well. Doug Keene drk@shcc.org Tue Aug 27

Specimen Preparation:
antibody penetration

One of my users is having problems with antibody penetration into frozen sections of brain from perfused (4% paraformaldehyde) mice. The mice are GFP+ and the GFP signal plus DAPI staining are fairly consistent through the depth of the section, so we know the optics are fine. However signal from two different primary and secondary antibodies (AF555 and 633) exists only in the top few μm of a 25-μm-thick section after 2 days incubating at room temperature; one antibody seems to penetrate a bit deeper than the other. Someone suggested we try 20% DMSO in the blocking buffer and in the antibody solutions, as she typically does that for whole-mount staining for better penetration. Any suggestions on what the problem could be and/or how to troubleshoot are much appreciated. ***Esteban Fernandez g.esteban.fernandez@gmail.com Wed Aug 28***

Usually penetration is more restricted for the labeled molecule (secondary antibody, protein A, streptavidin, etc.) than for the primary antibody since the labeled complexes are larger in size. In general, you want to look at such issues from two sides: from the perspective of the section and from that of the labeling molecules. Try to use antibody fragments rather than intact Ig, certainly for the label step. Whatever you may lose in binding stability is likely compensated for by more molecules penetrating and finding their targets. Use the smallest labels. Good penetration and good ultrastructure are achievable with ultra small gold particles, not so much with 10nm and larger. $NaBH_4$ treatment (1% in 0.1 M phosphate buffer, freshly prepared) in our hands helps ultra small gold Ig conjugates to penetrate in 0.5% glutaraldehyde fixed cultured cells. This might help also with your PF fixed material. Harsher treatments may be more successful but will progressively have an increasingly negative influence on structural integrity; however, sometimes one has no choice. Such treatments usually include taking up detergents in one or more steps of the fixation procedure. Most gentle is saponin perhaps which needs to be included during the immuno incubation steps as well. Triton-X-100 can be used as a 0.1% solution in buffer after the aldehyde fixation. When all else fails, detergent can be added to the fixative, but this results in general in serious extraction. The results need of course always be interpreted against the artifacts introduced by the applied procedures. Jan Leunissen leunissen@aurion.nl Wed Aug 28

Are your incubation times for the secondaries 2 days, or 2 days for the primaries and only 1–2 hours for the secondaries? Best approach is to incubate secondaries for the same length of time as the primaries. Glen MacDonald glenmac@u.washington.edu Thu Aug 29

Are you sure the primaries are all IgGs? I suspect you aren't using monoclonals since this is mouse tissue but a small percentage of monoclonals are IgM. And "poorly" made polyclonal sera can have a significant IgM component if they include antibodies from early bleeds. I also think anti-carbohydrate antibodies tend to be more likely IgM for some reason. IgM is, of course, 5× bigger than IgG. I have had this problem in my own lab with some homemade monoclonals. If the problem is from the secondaries, it should be easy to tell by switching the labels on the two different secondaries and seeing if the currently deep penetrating primary antibody is still detected. Tom Phillips phillipst@missouri.edu Sun Sep 1

Specimen Preparation:
chemical fixation of C. elegans for TEM

I have a question about chemical fixation protocol of C. elegans for TEM. I normally use high-pressure freezing (HPF) for C. elegans. Could I immobilize the worms inside an agarose pad and then add fixative? Would agarose cross-react with fixative? Some published protocols use agarose to help orientate worms after treatment with 2% osmium tetroxide. I would like to first put worms in agarose, then remove heads and tails and add fixative. For fixation, I would choose glutaraldehyde and osmium tetroxide because our lab used to use it a long time ago. ***Hong Zhan hzhan@live.com Thu Jul 25***

It is true that removing worms' heads and tails is difficult when they are awake. I tried using Levamisol to put them down first. I did *in vivo* probe labeling for target protein in worms, so that I want to try this way. But I have tried to put worms with drugs and embedded in agarose do CLEM protocol. I sealed freezing chamber with hexadecene, it was nice (according to Kolotuev et al.). However, I have some issues: after HPF (Leica HPM 100) fixation and embedding Araldit, the worm was cracked at some parts. I only have dorsal or ventral sides. I would like to know whether you have had the same problem and whether you have some tips for avoiding that? Hong Zhan hzhan@live.com Sun Jul 28

Partly in reply to Hong Zhan's question: I don't think cracks in HPF frozen material have been reported in specimens observed at a temperature below the devitrification or recrystallization temperature of water. Neither have cracks been described in freeze fracture replicas of high pressure frozen specimens. It is therefore likely that those cracks are the result of follow-up procedures. After HPF the available water is partly (at least) frozen to either high density ice or is in a high density amorphous state. These high density conditions are lost during freeze substitution. It is thought that high density forms have changed to low density already at the onset of freeze substitution and this would cause a change in volume at ambient pressure (from ~1.16 to ~0.92 g/cm^3), the tension giving rise to cracks. This has been suggested by Professors Moor and Daniel Studer amongst others, in their publications and presentations. Allow me to add a speculation: the problems may become more serious as a result of the embedding method, especially

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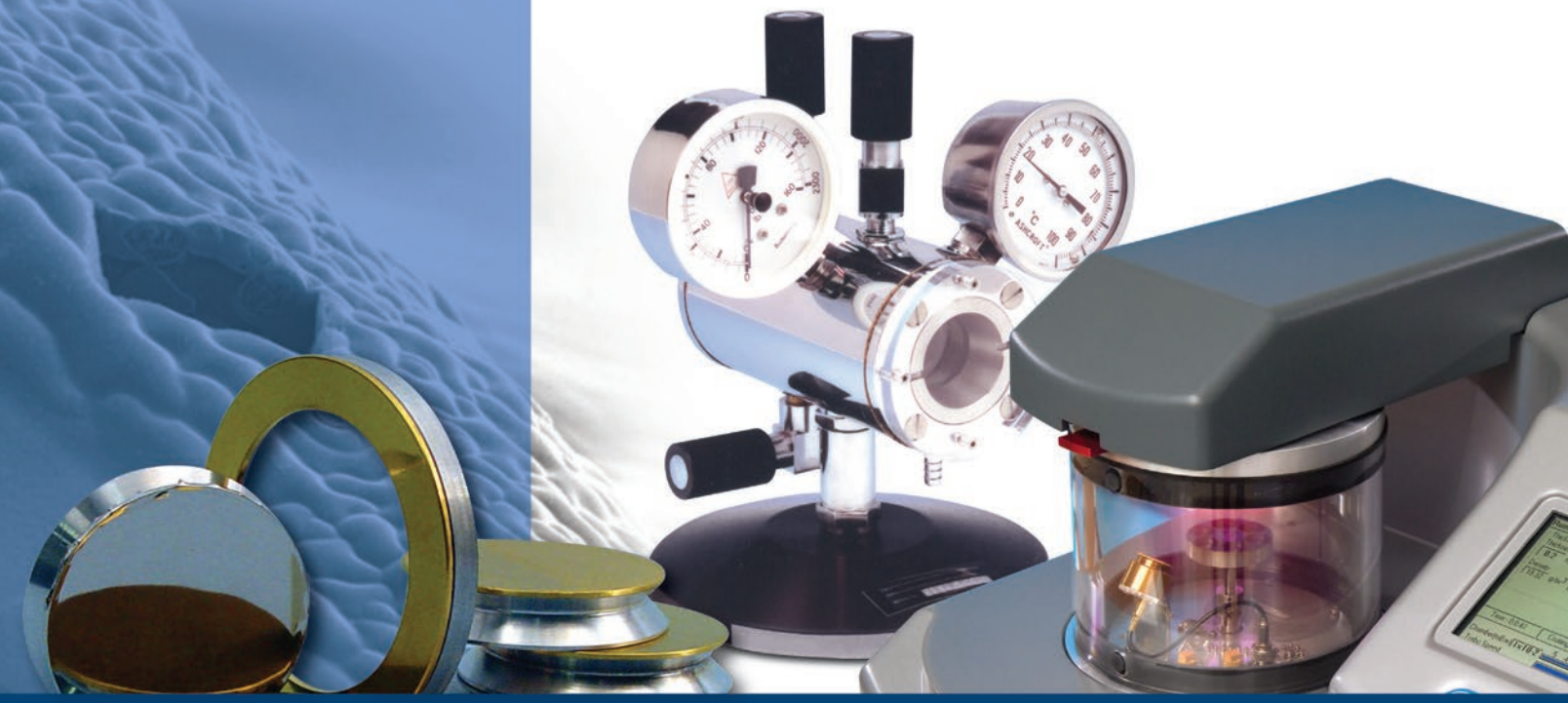

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when higher temperatures were used for polymerization. Proteins will change conformation as a result of elevated temperatures, especially if still hydrated. I am thinking of nicely curled up fried bacon! On a micro-scale this might very well lead to further cracking of pre-existing rips. But you asked if one can do something about this. Freeze substitution: physics can't be changed, and higher temperatures for freeze substitution are thought not to give sufficient removal of ice in a practical time span. I do not know if anyone tried to do this, by the way. The rate for removing water molecules from ice in a vacuum is not necessarily the same as in a medium. The solvent molecules will interact with water molecules as well and may bind water, possibly allowing for better removal rates than anticipated. Results obtained with Kent McDonald and Rick Webb's procedures support this. Having said that, one is bound to the freezing point of the medium of course. Embedding: people who have worked with lower temperature embedding methods may have had better results, with less pronounced cracks. I personally have no experience, but it seems a sensible way to go to minimize cracks. Jan Leunissen leunissen@aurion.nl Sun Jul 28

Specimen Preparation:
alternatives to uranyl acetate

Any thoughts on whether we can replace uranyl stains with Pt or other heavy metal stains? Our institution is on a safety/money-saving drive to replace all potential sources of radioactivity. Soon we will no longer use ^{32}P for DNA gels (and no more ethidium bromide either) and I am asked to get rid of uranyl acetate (UA). The radiation license is very expensive, as is keeping track of the radiation badges plus disposal of waste, so trying to save money as well as use safer chemicals. I am interested in others' experiences. ***rosemary.white@csiro.au Mon Aug 12***

We have extensively tested several alternatives to UA for both manual as well as automated thin section post-staining. A summary of these experiments can be found at http://bit.ly/VQSmJm. To cut the long story short, in our hands Oolong Tea Extract/lead citrate gave a rather weak stain, even using extended incubation times, and an increased risk of specimen contamination. The sections stained with Platinum blue/lead citrate were very clean and the stain was comparable with UA in density. For interpretation of the micrographs, possible variations in the distribution of the stain have to be taken into account, however. Negative staining is obviously an entirely different story, so we haven't gotten completely rid of UA to date. Here, I am very interested in the experience of others. Guenter Resch lists@nexperion.net Tue Aug 13

Besides the recent postings on MSA's-Listserver for Pt-blue staining and Oolong-Tea-Extract substitute for uranyl acetate, I would like to inform you of an article that recently was published in the *Journal of Electron Microscopy* (Toyko) that I found to be of interest also: Masamichi Nakakoshi, Hideo Nishioka, and Eisaku Katayama "New versatile staining reagents for biological transmission electron microscopy that substitute for uranyl acetate," *J Electron Microsc* (Tokyo) 2011 60: 401–407. Additional comment posted later: It is already known for years that e.g. lanthanum nitrate as a reagent (either added to the fixative or as separate incubation solution has some special effects on retention of elemental substrates in human tissue preparation. Replies to that post: (1) Yaroslav Tsytsyura—Thanks for this paper, we have already tried it and now switch to samarium instead of UA in routine protocols. Works both (en block and on grids) well. (2) Stefan Schöffberger—Electron Microscopy Sciences just introduced a ready mixed Uranyl Acetate Replacement Stain based on samarium and gadolinium triacetate. (3) Since I guess this post fits in here too, I would like to point to perhaps another possibility: non-isotopic (4%) Hafnium chloride in (100%) Methanol used as a contrasting/staining solution for ultrathin sections (Spurrs 60–80 nm thickness). Ikeda et al., 2011, "Enhanced effects of nonisotopic hafnium chloride in methanol as a substitute for uranyl acetate in TEM contrast of ultrastructure of fungal and plant cells," *Microscopy Research and Technique* 74:825–830. Thanks also to Stefan to tell us that the Sm-Gd-triacetate-staining will be/is commercially available now. And, last but not least: perhaps the old Bi-(bismuth) stain could be another alternative. Wolfgang Muss w.muss@salk.at Tue Aug 13

TEM:
unusual diffraction ring

I often see a weird ring in diffraction patterns when I do selected-area diffraction. We have a JEOL 2010 LaB_6 electron microscope. I talked to JEOL engineers, but they do not know the reason. Does anybody any insights on this problem? Or, does anyone know what's wrong? ***Yueling Qin yqin@buffalo.edu Sun Jul 2***

You are going to have to describe it better or post a link to an image of it. Prepare to describe your sample. An image of the area that you have selected would also work. Is it only happening with the one sample? Remember, the shape of the "spot" in reciprocal space will be "reciprocally" related to the shape of any phases that are diffracting. If you have a short dimension in the image of a phase, then it will be a large dimension in reciprocal space and the Ewald sphere intersecting this shape can look "weird." Scott Walck s.walck@comcast.net Sun Jul 28

We get that too, on our JEM 2100 LaB_6. In our case, I'm pretty sure it comes from the selected-area diffraction aperture. The ring shows up in a slightly different plane from the objective lens BFP, so it can usually be suppressed by changing the intermediate lens (diffraction) focus a little, but sometimes not completely, especially if the signal you are looking for is weak. I think it is a problem with the apertures JEOL uses. Not sure if these are Mo or Pt. I can't find an example at the moment, and we are waiting for the service engineer to come today to fix the instrument, but I can send you something for comparison in a day or two if it is working again. Phil Ahrenkiel phil.ahrenkiel@sdsmt.edu Mon Jul 29

When I worked at HP we had a very lovely JEOL 2500 TEM. Although the 2500 is a Field Emission Gun (FEG), we used to see something similar. Mr. Tamba, JEOL service based in LA fixed it for us. Rather, Tamba gave us the knowledge to fix it ourselves! We fixed it by always performing selected area diffraction (SAD) left of crossover, where the beam is more parallel. You know what they say about free advice ;0), but I believe that by operating left of crossover instead of right of, you shall get rid of your mystery ring! It is very easy to try, just give that intensity knob a little twist to the left. Pete Eschbach peter.eschbach@comcast.net Mon Jul 29

TEM:
oil diffusion pump heater alarm lamp

We have a TEM JEOL JEM 2000EX II, and three weeks ago, the alarm lamp indicating the breakup of oil diffusion pump heater was turned on. We have changed the DP heater with a new one and it seems to heat correctly, anyway the microscope continues to switch off after about 20 minutes from the starting and the DP heater alarm turns on again. In addition to the breakup of the DP heater, are there any other reasons for the DP alarm lamp to turn-on? Could the problem be more related to the poor grade of vacuum reached by the rotary pump? Thank you in advance for any your help. ***Alberto Fabrizi albertofabrizi.it@gmail.com Mon Jul 8***

You may find a thermal detector switch fixed on the DP. If the DP heater is operating correctly this contact is closed because the DP body is warm. But this thermal detector itself can be out of order. Be careful because there is another thermal detector fixed on the cooled area of the DP body. This one is to detect if there is enough water on the hose or not. Nicolas Stephant nicolas.stephant@univ-nantes.fr Mon Jul 8

TEM:
high tension problem

We have recently acquired a second-handed JEOL-2010 TEM microscope. Unfortunately, some problems seem to occur to the high tension (HT). The current emission quickly goes up to an abnormally large value when the acceleration voltage is slowly increased to a certain value (e.g., 50 kV). The HT tank also makes noise (sounds likes discharge). We have tried to manually increase the voltage extremely slow (1 kV/hour) to check if it will stabilize, but it fails to work. Any advice on the problem would be much appreciated. ***Yucheng Zhang zhangyc@gmail.com Wed Aug 28***

There are many problems that would give that which you outline but I believe this instrument has a gas filled high voltage tank so please check the pressure indicated on the tank. The operation manual should give you the correct pressure reading and how to top up the tank. Steve Chapman protrain@emcourses.com Wed Aug 28

TEM:
calibration grids

The recent emails on electron diffraction got me thinking. Overview: Can anyone let me know what substrates you have found successful for calibrating the scale-bar of cameras at high magnifications? Background: I have a 100kV TEM in which the standard etched line and cross-gratings are fine for calibrating our Megaview 3 camera in the range 100× to 70 k×, but higher than that it gets less precise as inconsistencies in the gratings become clearer and the number of lines in view become fewer (down to 1 line at 140k×). Problems: 1. More projects are working entirely at high mag looking at protein assemblies and fibrils etc. In addition, accurate measurements are important. 2. I have a 300 kV machine that has just been fixed after an extended downtime and high-mag tomography and negative-stain protein complex work will require good camera calibration. Questions: 1. Are there better gratings out there? 2. Do people use standard samples (e.g., negative stained TMV or like)? 3. Am I barking up the wrong tree and there is some diffraction method + sample that work better than direct measurement? ***Duane Harland duane.harland@agresearch.co.nz Sun Aug 25***

For many years prior to the microcircuit industry becoming involved in calibration standards we used the crystal lattice of suitable materials as a high magnification standard. Copper phthalocyanine (1 nm), potassium chloroplatinate (0.56 nm) and graphitized carbon (0.34 nm) are specimens available from the usual EM accessory providers and with the capabilities of modern instruments easily identifiable. Steve Chapman protrain@emcourses.com Mon Aug 26

There really is only one magnification standard to use for the TEM and that is the Mag-I-Cal sample. It can be used at all magnifications from the lowest to the highest on any TEM. In addition, it can calibrate the camera constant and image rotation. When you use other standards for the different mag ranges, you not only have the problem that you state, but often the results don't agree at the overlap range. In addition, some of them are beam sensitive. Scott Walck s.walck@comcast.net Mon Aug 26

SEM:
disabling auto-shutoff

I have an old JEOL 840A with a serious vacuum problem. I would like to be able to pump on the system with only a rough pump for an extended period for troubleshooting purposes. I know how to manually control the various pumps and valves, but the SEM still shuts off after 20 minutes if the diffusion pumps are not hot or if the internal pressure is too high. Is there a way to disable the auto-shutoff so I can pump with just the rough pump for a few hours or even overnight? ***David J. Wilbur david.wilbur@tufts.edu Tue Jun 25***

You need to be very careful about pumping on an SEM using only the rough pump if it is an oil sealed rotary vane pump. The reason is that these pumps can usually produce pressures well below the range of viscous flow (i.e., below about 10 Pa (0.1 Torr)). Under conditions of viscous flow backstreaming of oil vapors from the roughing pump cannot occur; however, below the range of viscous flow backstreaming becomes possible. Leaving a system standing for a long time while being pumped on only by an oil-sealed rotary vane pump can produce pressures in it that are low enough to allow it to become badly contaminated by oil vapors. Wilbur C. Bigelow bigelow@umich.edu Tue Jun 25

SEM:
error message

Does anyone have experience with a load current (LC) error on JEOL JSM-5800 SEMs or similar JEOL units? I recently purchased a used 5800, which functioned prior to moving. After moving, the unit starts up fine up to the HT ready point. When HT is pressed the LC error appears. This happens whether or not filament current is applied. The filament has been replaced and the Wehnelt cap has been cleaned. As a test, I removed the filament and still get the same error. The only reference to this error in the operating manual is under poor image quality troubleshooting. The manual lists the error as "fluctuate load current (LC)" and suggests cleaning the Wehnelt cap or replacing the filament. Currently no image can be obtained. I am thinking that something in the HT tank was damaged during the move. Does anyone have a drawing or schematic for the tank? Does anyone have a service manual for the JSM-5800 or similar unit? I am also open to troubleshooting suggestions and suggestions for repair. ***Fred Anderson fanderson@advantec-eng.com Sat Jul 20***

The problem is likely to be in the high voltage tank or the cable. To check out the cable remove the connection from the tank end (cover with foil) and check the LC again. Problem has gone then it's your cable which any organization working with high voltage (X-rays) should be able to help you at a much lower cost than the manufacturer. Steve Chapman protrain@emcourses.com Sat Jul 20

SEM:
beam/probe current measurement

I'm completely confused by the numbers I'm getting from my S430. My understanding is that beam current measures electrons in the column, and probe current measures electrons striking the sample. Probe current is less than beam current because things like apertures prevent all the electrons in the column from striking the sample. Now I would think that for a given column configuration, probe current and beam current have to increase or decrease together. I'm not seeing that, and I don't understand it. I've set up a data zone with both probe current and beam current displayed. When I increase or decrease the probe current, I see the secondary electron image get lighter or darker, as I expect, but beam current stays the same. Adjusting beam current isn't intuitive. I have to open a status box and add it as a data field, but then I can type it in. It seems the maximum beam current is 400 μA. When I change the value, the image gets darker or lighter as I expect, but the probe current doesn't change! To make matters more interesting, I can change the filament current, and it makes the image lighter or darker. I imagine as the temperature of the filament changes, its thermionic emission changes, causing more or less electrons to be released, hence a brighter or darker secondary electron image. However, wouldn't that change both beam and probe current? I change this and both beam and probe current as reported by the S430 stay the same. I am completely confused! These numbers on the S430 do not seem to match what I expect. In addition, my understanding is that one can measure probe current by shooting the beam into a faraday cup and measuring the resulting current as it leaves the stage. While I do not have one of these yet, I do see the ground for the stage coming through a feed through in the front door. What kind of connector is it, and where can I find one? It

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turns out the hackerspace has a Keithley electrometer that can measure picoamps, and I am guessing I can hook this inline somehow. That takes a BNC triax connector, which I would need to find. Any thoughts? ***Ryan Pierce rdpierce@pobox.com Sat Jul 20***

I think you may want to check the definitions. Probe current should be on the order of a nA. Beam current could be the filament heating current. I don't know the S430, so I cannot say. Changing the "beam current" could be changing saturation and should lead to some change in probe current but it would not be a direct change. It depends where you are on the saturation curve. Changing probe current is adjusting the condenser lens and is totally separate from what is happening in the gun. There would also be specimen current—that which is absorbed by the sample. That will depend on the sample and could vary for the same probe current. But like I said, I don't know the 430. Warren Straszheim wesaia@iastate.edu Sun Jul 21

I often use the Leica 430 and the Zeiss range of SEM on my courses, so I may be able to help you. Let us consider the parameters that should change the probe current, which we assume is related to the number of electrons striking the specimen. 1. Filament position—the further back the filament from the cathode cap the lower the emission level; known as beam current or emission current. 2. Bias Setting—the higher the applied bias field the lower the emission current but that is in relation to the filament position. For example with the filament very close to the cap the emission will increase unless the bias field is used to reduce that emission. Changing the current in the software simply changes the bias allowing more or less emission current. 3. The setting of the first condenser lens current, the higher the current the smaller the number of electron passed on from the first to the second condenser lens. Remember the probe is reduced in size, and therefore current, by the second condenser lens only taking the center of the beam presented to it, the remainder being trapped on the spray apertures 4. The size of the beam-defining aperture. In truth the probe current is a crude calculation that assumes a great deal. In relation to 1 above, the manufacturer assumes a specific filament position—probably totally untrue for most people's gun settings. In relation to 2 above with so many variables this seems to be ignored. In relation to 3 above this is the variable that is triggering a change in the probe current readout in the software. In relation to 4 above if the software asks for information on the size of this aperture they are using this data along with the first condenser current to vary the probe current readout. The manufacturer calculates the probe current when designing the instrument, assuming that the filament is set in a specific position in the cathode and the emission current is at a recommended value (setting the gun bias level). The software contains this information but apart from the change in first condenser and perhaps beam defining aperture no further software adjustments take place. You will judge from your experiments that what I relate is correct? If you wish to see the true probe current, where all of the variables I have discussed have an effect, buy a Pico Ammeter and set this up with a Faraday Cup using the "BNC" connector mounted on the stage door. I hope this helps because everywhere I go people ask the same question and I have to say "it's just a crude guess by the manufacturer." Steve Chapman protrain@emcourses.com Mon Jul 22

STEM:

probe changing shape

I am working on a JEOL 2100F recently while working on STEM I noticed that at very low mag the image observed tends to change to an oval shape, then after some time, it will become circular, then again oval in perpendicular direction. See link for images: https://sites.google.com/site/auxilliarylinks/ Why is this happening? Will it affect resolution? I want to do high-resolution HAADF but cannot attain lattice fringes. In addition, what is the best way to align STEM mode for highest possible resolution in HAADF? Anything I should specifically keep in mind? ***Amit Gupta amit.welcomes.u@gmail.com Thu Aug 29***

This is not due to probe shape changes. If it were, the resolution of your image would change across the field of view. Indeed, this happens at very low magnifications (you can see the stigmatism in the Ronchigram, especially towards the corners of the images where the displacement from the optic axis is large), but at such low magnifications you cannot detect the loss of the resolution. I suspect you are seeing the differential pumping aperture (DPA) that sits above the viewing chamber, just above the HAADF detector. I say this because the edge of the shadow is not sharp. The diffraction pattern you see while in STEM is formed by a real-space image of the probe and sample in the DPA. If the microscope is properly aligned, you should not see the DPA unless you work at low magnifications where the spherical aberration of the objective lens displaces the probe/sample image significantly. However, the elliptical nature of the shadow suggests several possibilities: 1. The STEM scan pivot points are not set up properly (does the probe move when tilting in imaging mode)? Indeed one, the excitation of the deflectors is not symmetrical. 2. You have a very highly excited stigmator (probably objective or diffraction stigmator). 3. The projector lenses are not at their correct (default) settings, i.e., the crossover in the DPA is slightly above/below the correct plane. Unless you're very familiar with the STEM alignments on a JEOL, I would suggest you have JEOL take a look at the STEM alignments. Jon Barnard jsb43@cam.ac.uk Fri Aug 30

The two images you show are taken at different magnifications and neither is actually very low. The circular shadow seen in Bright Field surrounding the image could be the limit of the illumination, try different condenser aperture sizes to see if that varies the diameter of the shadow, it's not important that you can see it but it shouldn't be oval shaped and shouldn't change direction, that's not right. What is intriguing is that the diameter of the shadow that I have assumed is the condenser aperture limit does not change with magnification in your two images (one image is 25K and the other is 60K) So the other thing to try is the camera length and perhaps the intermediate stigmator has a fault on it that can affect the shape of the illumination reaching the BF detector. The images look fine so the condenser and objective are doing their jobs alright and the beam on the sample is nice and round. Can you read the HEX values of the deflectors when the "fault" appears and see what changes? What kind of lattice images are you looking for and do you have an aberration corrector? Alignment can be as easy as using the HT wobbler and using the PL Align to shift the zoom point to the center of the detectors. What probe size are you using, how strong is your C1 lens; what illumination angle are you using, is it the optimum one? I'd get an engineer to come and check things out. Rob Keyse rok210@lehigh.edu Fri Aug 30

The images you showed are at different magnifications. The shape change of your STEM image you mentioned "… image observed tends to change to oval shape, then after some time it will become circular then again oval in perpendicular direction." Does it depend on magnification change or occurred at specific magnification? If the shape of your image is changing with magnification change this might be caused by bad alignment of STEM. I do not have any experiences with JEOL STEM systems, but in Philips CM12/STEM such behavior of STEM image was caused by bad eucentric position adjustment in Nanoprobe (STEM) alignment procedure. Oldrich Benada benada@biomed.cas.cz Fri Aug 30

Electron Diffraction:

angles

I'm looking for a simple estimation that I can't find in any textbook I've looked in, nor on the internet. I wonder if anybody can help me. If

I put a diffraction grating with spacing 1 Angstrom into the specimen holder and record an image of it. At what distance from the optical axis will the corresponding diffraction spot (for 1 Angstrom spacing) appear in the back focal plane of the objective lens? Assume a TEM with 200 kV and a twin lens, one that's typically used for protein structure determination. ***Philip Koeck philip.koeck@ki.se Fri Aug 23***

You simply need to use Bragg's law to calculate the scattering angle. Here is a simple table of Diffraction/Bragg Angles (in milliradians) as a function of D-spacing and Electron Beam Energy (80–300 kV). The actual distance you measure in your DP will depend upon your camera length. Also remember that the angular distance from the optic axis to the Bragg spot is twice the Bragg Angle. Just refer to any standard TEM text covering diffraction for the details. For 1 Angstrom d-spacing at 200 kV the Bragg angle is 12.54 mR.

d (Å)	80 kV	100 kV	200 kV	300 kV
	(mR)			
0.5	41.75	37.00	25.07	19.69
1	20.88	18.50	12.54	9.85
2	10.44	9.25	6.27	4.92
3	6.96	6.17	4.18	3.28
5	4.18	3.70	2.51	1.97
10	2.09	1.85	1.25	0.98

And a simple table of Energy to Wavelength Conversion:

Eo (kV)	Beta (v/c)	To (keV)	Lambda (Å)
10	0.1950	9.714	0.1220
50	0.4127	43.51	0.0535
100	0.5482	76.79	0.0370
160	0.6481	107.3	0.0285
200	0.6953	123.5	0.0251
300	0.7765	154.1	0.0197

Editor's note: The tables have been substantially truncated. The complete data sets can be found in the Listserver Archives.

Nestor J. Zaluzec zaluzec@aaem.amc.anl.gov Fri Aug 23

To follow up on Nestor's response … You can put a standard sample (such as an Al film) into your microscope. The d-spacings of Al are well known. Hence, using Bragg's Law, you can calculate the diffraction angles of the rings. You also have several objective apertures in your microscope with known diameters. If you put an aperture in over the diffraction pattern, you can compare the radii and get the absolute distance from the optic axis of the rings/spots at the Back Focal Plane. Also, knowing the diffraction angles and the objective aperture diameters, by simple geometry you can get a pretty good measure of the focal length of the objective lens. The distance from the optic axis at the viewing screen is, as Nestor said, dependent on the camera length. Note that the true camera length may be somewhat different from the microscope's indicated camera length. Henk Colijn colijn.1@osu.edu Fri Aug 23

Philip—For what purpose do you want to do that correlation, i.e., between the 1-angstrom distance in your real space of sample and the g-vectors, the "distance" of any diffraction spot? Remember, the correlation between a limited area of the direct space of your sample and the diffraction entities can be done by SAED, and the SA aperture is placed much lower in the column than the sample holder, i.e., the level of your real space sample. Besides, the correlation between the real space sample and the diffraction "product" is done via a Fourier Transform, and therefore there is no linear correlation. I have a question of mine, addressed to Nestor: how can one use the new kind of bars that are printed on diffractograms and show a number of 1/nm units, for the direct measurement, on that "plate", of the reciprocal space vector lengths ? What is the meaning of that unit, namely [1/nm]? I have difficulties in getting familiar with the use of it, so that I still use the accelerating voltage + camera length to "measure" the g-vectors, but that is a more laborious effort. Corneliu Sarbu crnl srbu@yahoo.com Sat Aug 24

Thank you all very much for the answers you sent. I just picked one out to reply to. Thank you, Nestor, for the tables. The reason I asked is that I want to know what spatial frequencies are removed by an objective aperture when recording high resolution (phase contrast) images. I wasn't sure whether the objective lens settings change when one switches between imaging and diffraction. Therefore that is the reason for the strange way of asking the question. Secondly I wasn't sure whether one can use the simple formulas given that the lens is not a thin lens. I don't know the position of the specimen with respect to the focal planes or the principal planes either. In any case everybody seems to point me to a simple calculation. For example for 200 kV and 1 Angstrom resolution cutoff I can take twice the angle in Nestor's table, resulting in angle = 25 mrad. I can also get that from Lambda = d sin(angle), if I regard the specimen as a two-dimensional grating. The next step is to use the focal length (not the distance between specimen and focal plane) as camera length, giving a distance of the spot from the optical axis R = 72.5 micrometers for 2.9 mm focal length (f). The formula is f * tan(angle) = R. Most answers seem to agree about using f. Please tell me if I got anything wrong. Philip Koeck philip.koeck@ki.se Mon Aug 26

Since you have clarified your question further I should also point out that the diffraction aperture is not always the limiting factor in phase contrast imaging particularly at the 1-Angstrom level that you seem to be interested in. When performing HR imaging experiments in this regime, one generally does not use any apertures. In HREM, you will need to invoke and understand the limits created by the aberrations (Cs & Cc) of your Imaging Lenses. Of these, the Objective Lens is the most critical. For high resolution work (nominally anything below about 0.3 nm) you will need to understand the Phase Contrast Transfer Function of your instrument. I recommend that you should pickup a good TEM textbook or review article that discusses High Resolution Phase Contrast Imaging. The book by Carter and Williams will be a good starting point. There are also several software programs available on the net which will calculate an approximate PCTF for you. See for example http://www.maxsidorov.com/ctfexplorer/webhelp/background.htm Just to give you a ballpark value, for a 200 kV FEI FEGTEM (Cs ~ 2 mm) without aberration correctors, the spatial frequency cutoff is in the vicinity of 0.2–0.3 nm. Nestor Zaluzec zaluzec@aaem.amc.anl.gov Mon Aug 26

I assume you are talking about the first zero of the CTF in (extended) Scherzer defocus. In structural biology we always work at high underfocus, so we have to go far beyond the first zero by some sort of CTF-correction and combining images with different defocus. The resolution range I'm thinking about is between 10 Angstrom and about 2 Angstrom so I think the size of the aperture should be important though maybe not in a completely straightforward way as Marin pointed out. I'm not sure if anybody has managed to get useful images of biological molecules without an aperture. Might be worth trying. Philip Koeck philip.koeck@ki.se Mon Aug 26

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