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Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 02/15/08 to 04/15/08. Postings may have been edited to conserve space or for clarity.

SPECIMEN PREPARATION – coloring Epon

I am flat embedding some very thin biological samples (they are grown on Aclar film and embedded). Normally it is very easy to find the sample; it is on the edge of the plastic. After I remove the Aclar film I put more Epon in its place and incubate it again. This works very well to protect the biology that is right at the air/Epon interface. The difficulty is that it can be very hard to find the biology. I was wondering if I could color the Epon (we are using Embed-812 - EM Science (#14120)) so that it would be easy to find the line between the colored and uncolored Epon? David Elliott <elliott@arizona.edu> Mar 28

I remember reading (but have never tried myself) that for specimens that are sandwich embedded between Aclar sheets, one can cut out the specimens that are good and rub a black wax pencil over the edges of the wafer before re-embedding. I can't remember where I read this, though. Maybe something from Peter Vesk or Jeremy Pickett-Heaps or more recently, in Microscopy Today? In your case, you might be able to circle the specimen? Andrew J. Bowling <andrew.bowling@ars.usda.gov> Mar 28

I realize that I was unclear in my question. I am not cutting the Epon face (in which case circling the biology would help), but I am cutting cross-sections. Thus when I am facing my block, I am looking down at the edge of the biology. I am trying to remove as much of the excess Epon as possible without losing the biology. Were I to write on the block face I would risk damaging the biology right at the air/Epon interface. David Elliot <elliott@arizona.edu> Mar 28

You are trying to indicate the interface, correct? A light sputter coating of the initial interface would work nicely eh? And will not harm the biology and would be very visible in the TEM or LM. Richard E. Edelmann <edelmare@muohio.edu> Apr 4

Some alternatives for coloring the unpolymerized "Topping" layer include: 1) Carbon particles from a carbon rod sharpener work very well and settle on to the interface. (You could try pencil dust or Hewlett-Packard laser jet toner. I know HP toner does not dissolve in resin but not all others do). 2) Using older accelerator results in very yellow/orange resins and may work for you. 3) A number of light microscopy "stain crystals" mix nicely into unpolymerized resin and result in weird colors: Fast Green, Crystal violet, etc. They are dry and some should not result in polymerization or sectioning problems (but to be honest I only used them for paper weights and desktop play things, I never sectioned them). The colors are not the "normal" colors I assume because they are not in an aqueous solution. Richard E. Edelmann <edelmare@muohio.edu> Apr 4

SPECIMEN PREPARATION – heat fixation of bacteria

We have been making slides to Gram stain in class. The book asks why they have to air dry and why the process cannot be sped up by gently heating them in a flame. Can you explain why this cannot be done? Second, can the slide be over heated during the fixing process? If so what happens? Judy Brennan <jlbrennan@comcast.net>

I always assumed heat fixing worked on two levels: it fixes the proteins etc.., it was found to work well, and is (or rather was*) very easy to do in the lab with a Bunsen burner. About three seconds is enough for this, over-heat the sample and you probably get just the carbon residue of what was bacteria (a Bunsen flame is 'hot') and possibly black smutty soot from the flame as well. Secondly heat fixing also kills the bacteria, which given the medical importance of most bacteria and their histo-stains, this is generally considered a good thing. Stains have to get through the cell wall and this is by diffusion (indeed differential diffusion through cell walls of different structure is the basis of the Gram stain). Heat the stain & slide, and the liquid solvent carrier of the stain might boil off the slide leaving a gunky goo on the glass and little stain inside the bacteria (this is a bad thing). Plus as http://www.fiu.edu/~makemson/MCB2000Lab/Exp2GramStain.pdf points out, just slight overheating during fixation can be a real problem and lead to false Gram stain results: "Note that the success of the Gram stain relies upon the integrity of the cell wall. Gram positive bacteria that have been overly heat fixed resulting in destruction of all or parts of their cell wall can appear to be pink (Gram negative) or have pink areas. This is an artifact! Further, old moribund cultures of Gram positive cells can appear pink. This is because the cell wall has allowed the challenge rinse to enter the cell. Successful Gram stains should be done on young, growing cells." The above links adds in more details on heat fixation and the Gram staining process as well. Some stains appreciate a bit of warming. Histology is as much an art as a science, and the more you do the better you get at it, even though the written method you are following often remains unchanged [and with classical colored stains, it was probably originally developed around 100 years ago]. Our labs have banned naked flames, so the Bunsen is out [hot plates/ovens are in]. Scientists can be trusted to build hydrogen bombs and anti-gravity devices for stealth bombers, but not with a schoolkid's Bunsen burner these days. http://www.microscopy-uk.org.uk/mag/indexmag.html?http://www.microscopy-uk.o rg.uk/mag/artjan05/mebacteria.html. Safety note: after working with any kind of bacteria, once finished, immediately place the slides into a disinfectant solution and wash, wipe work surfaces down with an appropriate disinfectant / antibacterial agent such as 70 % ethanol, sodium hypochlorite (aqueous, 10% sol), or a household disinfectant made up to the manufacturer's directions, and wash hands with an antiseptic soap. Avoid hand contact with the eyes or mouth whilst working with bacteria, and always handle cultures in the correct manner, assume everything is a pathogen. Keith J. Morris <kjmorris@well.ox.ac.uk> Mar 3

SPECIMEN PREPARATION – SEM of smooth muscle

I am currently attempting to view vascular smooth muscle cells and cardiac myocytes using a scanning electron microscope. I am fixing the cells with 3% glutaraldehyde/3% paraformaldehyde in 0.2 M sodium cacodylate buffer, 1% osmium tetroxide and dehydrating in a series of graded ethanol into 100%. Following dehydration, I am processing the cells in HMDS in varying concentrations (1:3; 1:1 and 3:1) in 100% ethanol and then leaving it in an incubator at 37C overnight to dehydrate. I am using HMDS as CPD resulted in large surface cracks and generally very poor preservation. While the HMDS appears to work better I am still not getting good results. The

https://doi.org/10.1017/S1551929500059319 Published online by Cambridge University Press

cell membranes of all the cells are being pulled off exposing the inner cell structures. Does anyone know of a good way to keep the cell membranes intact? Or maybe suggestions on what I might be doing wrong to cause the ripping off of the cell membrane? Kate Scholtz <kathrine.scholtz@students.wits.ac.za> Mar 15,

The one thing that pops out to me is that the ethanol/HMDS series should end with 100% HMDS, 2 or 3 times, and then air dry. After removing the water, all the alcohol needs to be removed. Ken Converse <kenconverse@qualityimages.biz> Mar 16

As previously mentioned, be sure to dry from pure HMDS. This usually takes 3 exchanges in 100% HMDS after the final 1:3 ethanol:HMDS. You might also try raising the drying temperature to 45 or 60°C. Another thing that will help the membrane preservation is first, reduce the concentration of glutaraldehyde. 3% seems rather high, try 1.25%. Next, add 1% monomeric tannic acid to the glut and/or the osmium. Tannic acid works well to help preserve membranes. Sometimes it works best in one or the other of the primary or secondary fixatives; sometimes it works best in both. Philip Oshel <oshel1pe@cmich.edu> Mar 17

One thing to remember is that after your HMDS treatment, you need to be careful to keep your specimen away from humidity. Specifically, if that incubator has Petri dishes or other moist things in there, your specimen may be partially re-hydrating as it sits in there overnight. In my experience, HMDS dries very quickly with no need for higher temperatures. I would suggest that you allow the sample to air-dry, at ambient temperature, for 5-10 minutes in a fume hood and then transfer it to a desiccant cabinet or a bell jar with nice, blue Dri-rite or silica gel. You can sort of wave the specimen at your nose periodically to see if the smell of HMDS is gone, and then move it to the desiccant (or straight into the coater). I think you are on the right track with high glutaraldehyde and OsO_4; however, ethanol (especially absolute) can be highly extractive of lipids, and you say you are having trouble with the membranes, so I would also suggest you also try to minimize your exposure to ethanol. If these are monolayers of cells on glass, you can go as low as 5-10 minutes on those ethanol and HMDS exchanges. Andrew Bowling <andrew.bowling@ars.usda.gov> Mar 17

I think that something is not right with the CPD processing; there is no reason that cells or muscle should have large cracks after CPD if done correctly. You may want to review the protocol and read the instructions very carefully and review the procedure with someone there who is very familiar with the equipment. There is some chance that the equipment, if automated, is not functioning correctly or that there is a misunderstanding about the process steps. Especially check the following: The final changes of ethanol (or amyl acetate, etc.) before going into the CPD must be absolutely free of water. The sample must stay covered at all times by fluid: do not allow the sample to "drain" and become uncovered by fluid at any point in the exchange of ethanol to liquid CO2. The Balzers CPD030 has instructions on the case to "drain and refill several times" but this should never leave the sample drained, always use multiple partial drain/fill cycles to remove the original solvent. Depending on the chamber geometry and amount of liquid CO_2 that you start with when beginning the heating phase, the sample could possibly "go dry" before the critical point is attained. Follow the instructions carefully; they usually state the starting level of liquid CO**2**. If too much, the pressure can become too high and blow out the protective burst-disc. Agitate the sample (depends on your system how this would be done) to promote exchange and full elimination of the ethanol (etc.). You should not smell ethanol (etc) when the final exchanges are made, or when you open the chamber at the end. Heat slowly in the heating phase. This is typically automatic, but should take ~15 min or more to reach the final temp (~38°C). Vent very slowly - should take another 10-15 min to come back to room temperature. This is often manual, so make sure you leak it slowly. Once out and properly dried, you must keep it dry; minimize handling in the atmosphere, especially if humid conditions. Be careful that glue or conductive paint solvents do not wick into the very dry and porous sample during mounting, again wetting it. There is no reason that you should have large surface cracks in this sample. Dale Callaham <dac@research.umass.edu> M**ar 29**

SPECIMEN PREPARATION – fungi for SEM

I have a colleague who would like to get some SEM photos of the underside of a mushroom that grows locally on the sides of trees down here in Florida. I was wondering what experiences people have had drying such a specimen. Air drying will surely distort the structure, but I don't have access to a critical point dryer. Justin A. Kraft <kraftpiano@gmail.com> Feb 29

I am not a mycologist. Don't let the fungus mature too far, or dry out or it may shed the spores. If it is important, keep some sample fixed and dehydrated to 70% ethanol (store in refrigerator or freezer) and maybe you can get access to a CPD unit later. There is a method for drying using hexamethyldisilazane (HMDS). This compound is available from the usual EM supply houses. It has a much lower surface tension than water and will probably do much better. You fix with aldehydes as usual, and postfix and dehydrate to 100% ethanol, immerse in HMDS for 5 min (small samples, longer for larger pieces), then drain and air-dry. There may be some special methods mycologists use. May be a good idea to en bloc treat with 2% aqueous uranyl acetate to add conductivity, or use one of the osmium-thiocarbohydrazide-osmium treatments. Be careful with the HMDS, it is unstable when mixed with ethanol, yet that is how it is used. Read the MSDS well. I had a bottle of waste that developed much pressure after sitting a while. There is another method for embedding in a wax that sublimes under vacuum, but I've never used it. I read that it is a mess for the pump, etc., but gentler. Dale Callaham <dac@research.umass.edu> Feb 29

In my experience, most fungal tissues do not do well in HMDS. Likewise plant tissues are all over the map- some work well, others not so. Vapor fixation over osmium crystal a couple of days in the fridge followed by slow air or freeze drying would be my first plan of attack, though admittedly I have not prepped the cap before. Scott Whittaker <whittaks@si.edu> Mar 1

You will not get good results if you don't CPD. I suggest you fix the specimens in glutaraldehyde or paraformaldehyde and keep them until you find CPD. If you wish, send the vials to me and I will prepare them for you, for free. Yorgos Nikas <eikonika@otenet.gr> Mar 2

SPECIMEN PREPARATION - cross section polishing for SEM

I've recently acquired an old Buehler lapping wheel and am experimenting with a few x-section techniques for SEM work. I am

https://doi.org/10.1017/S1551929500059319 Published online by Cambridge University Press

using a final polishing cloth and 0.05 µm colloidal silica for the final step and the suspension is sticking to the metal wiring. Despite my efforts to clean the surface (with acetone, dd water, etc.), I can't free the surface of the remaining suspension material. Any advice in regards to freeing the surface of the sticky material will be greatly appreciated! Also, I read the recent inquiry and a few of the responses in regard to the removal or the Au/Pd coating for a biological specimen. What does one recommend for the coating removal for a material sample? Marissa Libbee <mlibbee@gmail.com> Mar 4

It is very important to not let the colloidal silica dry on your sample. Once it dries, it becomes very difficult to remove. South Bay Technology (www.southbaytech.com) does sell a sample cleaner that works very well at removing colloidal silica from samples. Try contacting them; you may be able to get a sample for testing. Of course, I think a bottle of the stuff is only about $10 so you may as well just buy some! David Henriks <henriks@cox.net> Mar 4

I know what you mean! David Henriks is correct, do not let the co-Si dry on the sample. What I do is rinse the sample very well with running deionized water, then add a drop of soap/detergent solution to the sample surface, then scrub gently with a cotton swab saturated with the soap/detergent solution, rinse again in running deionized water and then dry with clean (no oil!) compressed air or dry N2. I use dry N2, but my building is plumbed with it. The scrubbing motion is rolling the saturated swab over the surface once or twice. For the soap/detergent, I use Micro-Organic soap from Allied High Tech, but Joy (or any other you might have) dishwashing liquid works well, also. One or two drops in about 25 mL of deionized water is a good mix. (Alconox would probably work, too.) You just need something to break the surface tension of the water. For Au/Pd removal from a materials sample: if it's a cross-section face, just re-polish it. If your sputter coater has an etch capability, that is good also. For other things, you can try using KI+I2 etch. Recipe: 2.3 grams potassium iodide, 0.65 grams of Iodine and 50 mL of deionized water. Stir until all solids are dissolved. This keeps almost indefinitely in a glass bottle. Be aware that this etch will attack Al and Cu to some extent. If you are coating something for the SEM that you know you will need to do something else to, I recommend using carbon (if you have it). That will come off with an oxygen plasma in just a few seconds with no damage to your circuitry. Becky Holdford <r-holdford@ti.com> Mar 4

Just one thing to add to the other replies, which I didn't see in them: colloidal silica will precipitate (i.e. stop being colloidal) if the pH becomes at all acid. So the thing to do is to remove all the silica before this happens. Also, if the solution dries, the silica will agglomerate. A mildly alkaline detergent works well - I usually use Decon 90 - and I wash/clean the sample in a weak solution straight off the polishing wheel, before it sees any deionized water or other cleaning agent. Richard Beanland <rb258@hermes.cam.ac.uk> Mar 5

SPECIMEN PREPARATION - Labeling SiO_2 particles

I am working with someone in the materials section here who is embedding silica particles in a solid matrix, and would like to be able to follow their distribution. The particles are SiO_2 and must be monodispersed as he sets up the reaction. The linker in the matrix is a silane derivative. It had occurred to me that it might be possible to label the particles with a fluorescent dye, and visualize them either with a confocal or widefield instrument. Oh yes, the particles are about 0.5 micron. Any suggestions of ways to label the particles? Joel B. Sheffield <jbs@temple.edu> Mar 11

I recommend embedding the silica in LR White - hard grade - without accelerator. Ignore all the instructions about accelerator: the accelerator is neither needed nor desired for your prep since interference of curing of the resin by oxygen is not an issue when cured at high temperatures and one does not want to use an accelerator in heat curing. Place particle/resin suspension in open embedding containers of your choice, vacuum degas, and cure overnight at 80-90°C in a nitrogen-purged oven. You will get a hard, brittle block, ideal for cutting the silica. You are on your own about creating a monodisperse suspension of particles in the resin. If you find a good way to do that, please reply to the listserver or to me off-line. Gary M. Brown <gary.m.brown@exxonmobil.com> Mar 12

SPECIMEN PREPARATION – quantum dots for EM

I am looking for reference papers on quantum dots application for electron microscopy. Has anybody used QD for EM? Dorota Wadowska <wadowska@upei.ca> Mar 28

Bazett-Jones (http://www.sickkids.ca/bazett-joneslab/default.asp) has done some work with Quantum Dots using an energy filtered TEM. See: Nisman R, Dellaire G, Ren Y, Li R, Bazett-Jones DP (2004) Application of quantum dots as probes for correlative fluorescence, conventional, and energy-filtered transmission electron microscopy. J Histochem Cytochem 52(1):13-8. Larry Ackerman <larry.ackerman@ucsf.edu> Apr 7

SPECIMEN PREPARATION - immunogold staining of DNA and chitosan

Does someone know if one can perform immunogold staining for DNA and chitosan polymer or if there are other methods for staining DNA and chitosan in electron microscopy? Monica Nelea <monica.nelea@polymtl.ca> Mar 10

Colloidal gold has been used for the detection of DNA, but bear in mind that the size of the probe (gold particle and whatever other macromolecules are attached to it) is significantly greater than the size of the DNA molecule; therefore precise localization is difficult, if not impractical. Another method for imaging DNA is platinum shadowcasting. Daryl Meyer <dameyer@wisc.edu> Mar 10

The terminal deoxynucleotidyl transferase (so called Tdt) method is an elegant technique to detect DNA strands in TEM. It is very sensitive, following its author Marc Thiry. It is based on an enzymatic reaction; this is not a direct immunogold detection. However, you probably won't be able to reproduce the technique just by reading the papers of the above mentioned author. Stephane Nizets <nizets2@yahoo.com> Mar 11

MICROTOMY - picking up cryosections

I have managed to freeze the samples, get them into the chuck of the microtome and trim them, I even get sections but I can't get the sections from the edge of the knife to the grid. They end up as little snow balls. I've been trying with an eyelash to lift or flick them over. Does anyone have a tip as to how this can be done reliably? Bob Harris <bharris@uoguelph.ca> Mar 4

There are a couple of factors that may be causing your problems. 1. Static electricity - are you using an ionizer? 2. Speed of cutting - Either max out the cutting speed and pull off ribbons,

https://doi.org/10.1017/S1551929500059319 Published online by Cambridge University Press

catching them on an eyelash as soon as they begin forming. Or use a micromanipulator (Ladinsky, 2006) and lower the cutting speed to 0.1-0.2 mm/sec. KD Derr <kderr@nysbc.org> Mar 4

I use a loop, dip it in my cryoprotectant (2.3 M sucrose with 10% PVP in my case) and lift the sections on the liquid. Then put the sections (and the drop of liquid) on the grid (make sure the grid is not too hydrophobic). I then invert the grid and float it on a dish of my cryoprotectant. The sections don't desiccate that way and when the section is picked up on the liquid-filled loop they spread out. David Elliot <elliott@arizona.edu> Mar 4

Try the Tokyasu method: use a small platinum loop that has been dipped in 2.3 M sucrose, hover it near the knife until it just starts to gel, then press the sucrose against the sections. Remove the loop from the chamber and allow the drop to thaw. Now, press the bottom of the drop against your grid to transfer the sections there. If you need to keep the sections frozen, this method is no good for you, and I must defer to others who deal in the world of continuously frozen stuff. Lee Gould <lcgould@med.cornell.edu> Mar 4

Strangely enough for all the precision engineered and expensive equipment required for cryothin sectioning, it all comes down to the pickup. For my two bits, when I get a ribbon or a single section laying down on the knife face I use a platinum wire loop dipped in 2.3 M sucrose in 0.1M phosphate buffer for high compression tissues such as skin (this allows them to stretch back out) or a mix of 1 part 2% methylcellulose and 2 parts 2.3 M sucrose for low compression tissues such as thymus (this keeps them from stretching). It seems to work best for me if the droplet is fairly flat, not bulging with pickup solution. The pickup for sucrose must be fairly quick, about 2 seconds. If the droplet is frozen when it contacts the section, the section will have poor morphology. The drop should still be jellylike where it is cold enough that it doesn't stick to the knife face like syrup. The methylcellulose mix gives you more time (perhaps three seconds) and gives you the added feature of smoking (vapor) when you put the loop into the cold chamber. The section should be picked up right when the vapor stops. In both cases the pickup should be a quick, smooth and a gentle press of the droplet against the sections. Don't hesitate and allow the sections to fly up toward the loop (this might be the snowball). By the time the loop is retrieved, the droplet will be frozen. Have your grid (face up) on a clean surface and after waiting about 10 seconds for thawing, slowly contact the grid with the loop. The sections will adhere to the grid. I usually continue straight to immunolabeling so I gently place the grids face down on my solution of PBS with 0.5% BSA for the subsequent immunogold. Robert Underwood <underwoo@u.washington.edu> Mar 4

There is an assumption in the replies that the poster is working with fixed and cryoprotected material. However, there is a clue in the original message that suggests the specimens may be rapidly-frozen and fully hydrated ("I have managed to freeze the samples, get them into the chuck of the microtome and trim them"). If the specimens are unfixed and not cryoprotected, none of the retrieval methods will work. However, using an anti-static line (deionizer) should help spread the sections out, but only if the knife is very sharp (and perhaps a diamond). The correct temperature is also important for sectioning un-fixed, un-cryoprotected material. I think the cryochamber should be held around -125 degrees for sectioning vitrified specimens, but it has been a long time since I did any of this. Paul Webster <pwebster@hei.org> Mar 4

IMMUNOCYTOCHEMISTRY – permeabilization

We have been starting to run pre-embedding immunogold experiments. For monolayers of osteoblasts, we have used 0.05% Triton-X-100 as a detergent for permeabilization with mixed results. I was wondering what detergents and concentrations others have employed successfully. Also, I was wondering if there are any detergents that can be used for the detection of membrane-bound antigens. I have heard that saponin is a less harsh detergent and that it must be used in every step of the procedure, but I do not know what concentration is recommended. Shannon Modla <modla@dbi.udel.edu> Feb 19

We use 0.1% Saponin. It does not destroy the ultrastructural appearance of the membranes; however the antibodies do not penetrate very deep into the tissue so I cannot say whether it is very effective. Gerd Leitinger <gerd.leitinger@meduni-graz.at> Feb 19

I would like to thank those who have responded to my inquiry. Currently, the suggestions were: 1. 0.5% Triton-X-100 for 5 min in PBS; for saponin, use 0.1% in PBS. As a reference, refer to Humbel BM, De Jong, MDM, Muller, WH, and Verkleij AJ. 1998. Pre-embedding immunolabeling for electron microscopy: An evaluation of permeabilization methods and markers. Microsc Res Tech 42: 43-58. 2. 0.1% saponin produces improved ultrastructural preservation but poor antibody penetration 3. New Triton-X-100 at 0.01% during the block only. Incubate in primary overnight. For saponin, use 0.05-0.1% in every step. 4. 0.05% Saponin in every step. Saponin basically sits between (intercalates) cholesterol groups on the plasma membrane, once removed those "holes" seal up. The deeper you get into a cell (less cholesterol groups) the less permeabilization you get and hence labeling. As a preface to my original question, we are using ultrasmall gold conjugates from Aurion and the Aurion SE-EM silver enhancement kit. We were attempting to perform a double-labeling experiment by using different enhancement times for the different primary antibodies. Both primaries are raised in the same species so labeling had to be performed consecutively. After the final enhancement, the samples were briefly postfixed with 0.5% OsO_4 for 15 min. We used the following paper as an outline: Yi H, Leunissen JLM, Shi GM, Gutekunst CA and Hersch SM. 2001. A novel procedure for pre-embedding double immunogold-silver labeling at the ultrastructural level. J Histochem and Cytochem. 49: 279-284. The initial results showed a variation in the size of the particles such that the variation was more of a continuum rather than two discretely sized populations. There are numerous explanations for the negative results such as poor antibody penetration, a problem with the silver enhancement, or experimental error. To test the silver enhancement reaction, I adsorbed secondary antibody on poly-l-lysine coated grids and performed the enhancement. The outcome also showed a wide variation in the size of enhanced particles, leading me to wonder if this was normal. Although statistics were not performed, certain sized particles did seem more numerous than others, but the variation was still surprising to me. Therefore, this brings me ask more questions: Is there a way to make the silver enhancement reaction more uniform? Is the variability in the enhancement a result of a variation in the size of the ultrasmall gold conjugates or a variation in the enhancement reaction itself? Are there other silver enhancers/ gold enhancers

https://doi.org/10.1017/S1551929500059319 Published online by Cambridge University Press

that produce a more uniform reaction product? Has anyone else had success performing double-labeling using ultrasmall gold and silver enhancement and if so are there any tips or tricks? Shannon Modla <modla@dbi.udel.edu> Feb 19

I have seen some excellent ultrastructure in preparations utilizing ethanol for permeabilization of tissue. Usually 50% ethanol was used for one or possibly two hours. However, the percentage of glutaraldehyde in the primary fixation is a significant factor. Good results can be obtained with as little as 0.1% glutaraldehyde and if you are lucky 0.05%. For a monolayer of cells I would suggest 15 to 30 minutes of 50% alcohol. There are many protocols described in scientific literature and so many techniques and variations you could spend the rest of your life trying them! Here is one exemplary reference with very nicely detailed protocols: Llewellyn-Smith IJ, Dicarlo SE, Collins HL, Keast JR (2005) Enkephalin-immunoreactive interneurons extensively innervate sympathetic preganglionic neurons regulating the pelvic viscera. J Comp Neurol 488(3):278-89. Larry Ackerman <larry.ackerman@ucsf.edu> Feb 19

The homogeneity of gold/silver particles is influenced by a range of factors. Size differences of the gold particles and the quality of silver enhancement are only one side to the story, but more importantly the specimen and the particular enhancement conditions play a role that should not be overlooked. And as a last factor, inhomogeneity may also be related to fusion of gold/silver particles during the enhancement process. As far as the aspects of reagents and procedure are concerned, uniformity of the diameter of the gold/silver particles using Aurion R-Gent SE-EM may be improved by slowing down the formation process using a developer with an initiator:activator ratio of 1:60 (instead of the 1:40 ratio that is in general sufficient). This change needs to be compensated by an increase in enhancement time. Next to the paper by Yi et al, we would like to cite the impressive paper by Ravi C. Balijepalli et al. (2006) PNAS 103(19): 7500-7505. Jan Leunissen and Peter van de Plas <leunissen@aurion.nl> Feb 26

IMMUNOCYTOCHEMISTRY - colloidal gold

I vaguely remember a publication on this subject where the author(s) mentioned the size of the epitope, the dimensions and shape of primary and secondary antibodies (IgG) and the gold particles that seem to explain the localization of gold particles to the side of the epitope (structure). Can you give me the citation? Wen-Lang Lin Feb 29

Simmons, S.R. and R.M. Albrecht. 1989. Probe size and bound label conformation in colloidal gold-ligand labels and gold-immunolabels. Scanning Microscopy Suppl. 3:27-34. Daryl Meyer <dameyer@wisc.edu> Feb 29

One such paper is Drenkhahn and Dermietzel 1988 Journal of Cell Biology 107:1037, but there are others as well. Rosemary White <rosemary.white@csiro.au> Mar 2

IMAGE PROCESSING – false color images

Please could you advise me of the best way to produce false-color images of black & white digital SEM captures. Is there specific software for this, or is it generally done in something like Corel PhotoPaint (which I have experimented with)? Anthony Butcher <anthony.butcher@port.ac.uk> Feb 16

Coloring images depends on various things and can be easier or harder to do. The easiest way is having not one but two or more detectors on the SEM to use during acquiring an image. These different B&W images can be converted to RGB files with Photoshop or any other image editing software (ImageJ, ...), attributed with a chosen color and mixed. This will give you a two- or more (numbers of detectors) colored image, including all the mixed colors of the colors chosen for each B&W image. Most of the images on the following sites are made with only two SE / BSE detectors: www.elektronenmikroskopie.info/ausstellungen/wuerzburg (please give the site some seconds to load and then do right clicks to see further images) and www.quantifoil.com/calendar_ .pdf. Sometimes I used paths to alter parts of the image in color (background etc.) Normal way would be having only one B&W image to start with. Here you need to do a lot of work, basically starting with converting to RGB and changing to a color fitting your specimen. Next you need to manipulate parts of the image using paths or masks. I am accustomed to Photoshop, but I think any other editing software where you can work very precisely with paths, masks, etc will do it in color and tonality. This will mostly lead to a more "artificial" re-inventing the specimen you had as a B&W image. Normally - unless you work very precisely - it will not look natural. So: best would be to use multiple detectors and a scanning electronic like DISS5 from www.pointelectronic.de (...only satisfied customer...), which enables you to use max. 4 detectors parallel, use one detector for the basic color, the other to use as colored spot-lights. Stefan Diller <stefan.diller@t-online.de> Feb 16

It is not clear to me what you mean by false coloring. Color assignment is done for a variety of reasons. If you mean the way people show a bacterium as <shades of green> in an otherwise grayscale image, better to let others help you with artwork. However, keep in mind that this is artwork and please see the Rossner and Yamada paper (link at the end). SEM images are inherently just intensity data - a binary value representing the intensity of "signal" for each pixel position. This is traditionally "monochrome" or grayscale but color can be assigned to represent each intensity as well. It is easy with ImageJ (http://rsb.info.nih.gov/ij/) to assign a LUT to a grayscale image. This does not change any values, just substitutes colors for the grayscale value. To the extent that the colors or color mapping may not be, or be perceived as, linear intensity, this can be deceptive if used improperly. As Stefan Diller pointed out, if you have different detectors and thus different information, these different views can be used to map an image in color, distinguishing the features revealed by those detectors. Another reason for using color in SEM images is to show depth - if one (grayscale) image is taken and then the sample is tilted some degrees and another is taken, these images can be assigned to the red and green (or red and blue) channels of a RGB image. Depending on the software used, it either uses zero values for the blue channel, or an image of zero must be supplied for the empty channel. The RGB image, viewed through R-G (or R-B) glasses allows each eye to see only one view and the brain "sees" the depth. If you are getting started with this, the link for Digital Imaging at the Molecular Expressions website is a good place to start for reading on digital imaging (including manipulations): http://micro.magnet.fsu.edu/primer/digitalimaging/index.html But also very important reading if the result is for publication is the Rossner and Yamada paper in JCB (available as pdf): http://www.jcb.org/cgi/content/full/166/1/11. Dale Callaham <dac@research.umass.edu> Feb 16

Some call false color "pseudo color." Either way, it is the conversion of a grey scale image to color. The process can be done by combining color separations as B&W images from separate detectors or by using a look up table (LUT) for grey scale values to color. Combining detector images can be done using Dindima Spectrum while complex LUTs can be created with Maxim DL. Alternatively, one can use Photoshop to create layers with different colors of accentuation based on grey scale intensity. The LUT methods typically require a post processing noise filter like Gaussian or median. The multiple detector images do not. If you take one image with one detector and another image with another detector, they likely will not be exactly aligned. An FFT alignment routine will bring them together. SIS analySIS Opti will do this. Some examples of software colorization of SEM pix can be seen at: http://www.photoweb.net/pw_gal_macro/gal_microphotography.html Some of these pictures are somewhat aggressively compressed and display artifacts of compression. Try to ignore this. Gary Gaugler <gary@gaugler.com> Feb 16

IMAGE ANALYSIS

I am doing TEM on several cell cultures. The investigator is sure that some cell lines "appear" to have many vacuoles and other cell lines "appear" to have few vacuoles or none. The investigator would like me to quantify this condition. My question is how does one quantify this in such a way as to be statistically meaningful? Secondly, the investigator feels that some cell lines have more mitochondria, while other cell lines have fewer mitochondria. Again, they would like to get quantitative data of this condition. What are people's thoughts on how to approach this question? If any respondent is curious as to what I have already tried to explain to the investigator please include your telephone number and I will gladly call you. All help is appreciated. Tom Bargar <tbargar@unmc.edu> Mar 25

There are two ways that I have used. One involves serial sectioning though cells (Elliott in Microscopy Today, Jan 2007) and reconstructing the cells. That way you know how many/how large/what shape the vacuoles are (Elliott et al, PNAS). The second way is to use the well worked out tools of stereology. The book I like for this is "Unbiased Stereology - Three-Dimensional Measurement in Microscopy" which is available from Amazon for about $18. David Elliott <elliott@arizona.edu> Mar 26

If you want to "go microscopic", I agree that stereology is probably the way to go, but I don't think that you need to spend the time and energy (and expertise) for 3D reconstruction. In the end, you just want to know if the vacuoles are more numerous/bigger, you do not really care to know exactly their volume. You have the chance to work with cells in culture, which means that the cell population is relatively homogenous. Cutting through a cell pellet or through flat-embedded monolayers will give you plenty of cells to count and measure with joy and excitement. Flat embedding would probably offer the advantage that most of the cells in one sample will be cut at the same level and the cell area (in sections) offered for analysis will be in the same order. Flat embedding also preserves the cell morphology in situ. However to embed and cut monolayers is a little more complicated than to collect and pellet the cells. Another problem with flat embedding is that you may cut right where the vacuoles are in one sample and in the other, cut in a part of the cell layer where the vacuoles are not. In this regard cutting through pellets will give you fully randomized cutting directions in all cells. The principle here is to photograph randomly or systematically many cells, to measure the total cell surface and the surface of the compartment of interest (vacuole for example) and to make statistics. The statistics will tell you if your result is significant or not. The good news is that you can quantify both the vacuoles and mitochondria with the same set of pictures. It would probably be worthwhile to collect their number as well as their surface (which could give you the mean surface for example.). If you are not familiar with stereology it would be worthwhile to read some book because this technique, like others, has its pitfalls and one can easily produce biased results. However, once correctly performed, it is pretty easy. This is the EM solution. Another much faster solution, but I have no experience in this field, would be to find a fluorescent dye for vacuoles and mitochondria, and to FACS the cells. I am confident it should be pretty straightforward, just talk with people of the field. Stephane Nizets <nizets2@yahoo.com> Mar 27

TEM camera problems

Recently, we have bought a new 200kV TEM with a 2k CCD. When we acquire an image in full frame mode, we observe that five pixels at the very ends of the CCD are merged into one. This repeats through-out the circumference of the CCD. So, my image has 2038x2038 pixels of useful data. In half and quarter mode acquisitions we do not see this problem. Previously, I was using 1k CCD's and never experienced anything like this. Is there some kind of technical issue with 2k cameras that they have to give-up five pixels around the CCD? Ayten Celik-Aktas <celikaktas@gmail.com> Mar 3

It might be a problem of image clipping. Try to go through the camera and image acquisition setting in your camera software and look for "clipping border" item (or something similar) and set it to "No clipping". Oldrich Beneda <benada@biomed.cas.cz> Mar 3

I can confirm that we also have set the outermost pixel to an average value. For our CCD camera model, this greatly improves the 'quality' of the power spectrum, by the elimination of sharp edges. This feature needs to be set for each camera resolution, so for other settings, it may not exist. Reinhard Rachel <reinhard.rachel@biologie.uni-regensburg.de> Mar 3

There are often bad pixels at the edge of CCD chips. With our 2k camera, the 8 pixels nearest the border contained enough bad pixels that the camera was configured to set them equal to the values of the pixels just inboard of them. If you have a Gatan camera and DigitalMicrograph, there is a panel you can access that lists the pixels so designated. I'm pretty sure that the same is true for Tietz cameras and software, but I have no experience with them. Bill Tivol <tivol@caltech.edu> Mar 3

FESEM - exploding ceramics

I was just imaging a carbon coated piece of ceramic in our FESEM under normal conditions (dry, 0.8 kV, 5mm WD) and switched to a higher kV. Nothing I have not done before. Much to my surprise, the piece exploded, knocking three other samples off their respective stubs, and reducing the piece I was looking at to a pile of dust. This is a first for me. Has anyone experienced this before? Can anyone offer an explanation so I might avoid this in the future? Derrick Horne <dhorne@interchange.ubc.ca> Feb 26

I've seen the mineral Lawsonite do this (very impressively) under the electron beam. I believe the term is "decrepitation".

This mineral contains a water molecule locked inside the crystal structure which might have something to do with it. John Donovan <donovan@uoregon.edu> Feb 26

I had two such "explosions" of samples. Once it was with an organic crystal, containing much OH, which slowly dehydrated under the vacuum and cracked. It was too carbon-coated, and suddenly it exploded though the charge accumulation in the cracks. Another time it was with a very bad sol-gel ceramic, where the grains were badly sintered. Charge accumulation dissociated the grains. One could see grains flying away individually until the sample itself exploded. In the first case, the dehydration caused cracks, which disrupted the carbon film, and charge could accumulate between ungrounded parts, which pushed back on themselves. In the second case, the carbon coating, as it is a point source evaporation, doesn't give a continuous conductive film, between the grains. Same effect as with the former. And as grains fly away, the effect became more and more dramatic! The ways to avoid this would be: what you have done, low voltage SEM, at the "right" primary energy (not always easy to find, and changing from one place to another). If the sample is porous, multiple carbon coatings, with the sample tilted in different orientation in respect to the source. Or use magnetron Ir, Cr, Os coating, which is less directive. Low-vacuum/ESEM, where the charge cancelation is better done than in high vacuum SEMs. But, this is not compatible with FE-SEM for high resolution. It depends of the magnification you need. Jacques Faerber <jacques.faerber@ipcms.u-strasbg.fr> Feb 27

ESEM – filament degradation

I was curious when a filament starts to degrade if you see the effect larger at high or low kV. We have an ESEM that we are still trying to learn taking wet samples. At 5 kV to attempt less damage to the cells I can't seem to get a focus to clearly distinguish features less than 1 mm. Then I tested imaging a normal sample under high vacuum with the low kV and still could not get a good focus on the sample. I do not remember ever using that low of a kV in high vacuum before but I thought even at 5kV you should be able to get a better focus than what was achieved, and at 30kV I was still able to get a clear crisp image on the order of 1 micron-100nm. Articles pulled from a few journals show other users with similar scopes getting clean images on the scale of 10 microns. Is it possible I am doing something wrong or might the filament just being aging? It has been in the scope for 46 hours and our normal lifetime seems to be around 50 hours. Jason Saredy <sarj0007@unf.edu> Feb 16

Just as an update. Yes, the apertures were replaced recently, the normal procedures for focusing are known on this end and maintenance as well. The normal sample referred to in the original post referred to a TEM grid with gold flakes. Thanks for suggesting re-saturating the filament at the lower kV. Readjusting some things this morning it was noted how the stigmator didn't appear to be affecting the image until it was set to an extreme. The images started to come in decently under high vacuum, but it leaves no room for fine tuning of the stigmator. So alignment problems, perhaps with the lenses? And I am fairly certain we are not oversaturating the filament, but several members of the listserv commented our filament lifetime was much shorter than normal. Jason Saredy <sarj0007@unf.edu> Feb 16

I would like to comment on Jason Saredy's problems. I believe some of the answers that he has had could be a little misleading, but I will explain. Listers have commented that Jason's filament life (around 50 hours) could be a clue to his problem of not being able to obtain good quality images at 5kV. I feel this is a misunderstanding of the role that the filament plays in performance. In Microscopy Today, March/April 2003 was an article called The Life and Death of the Tungsten Hairpin Filament, where I discussed the operating advantages and disadvantages that would be stimulated by the filament. In fact it is the position of the filament that varies its contribution towards resolution. The manufacturers generally guide operators to a filament to cathode distance that offers reasonable resolution with a reasonable filament life; the 50 hours that Jason was getting fits in with this criterion, I would say 50 to 60 hours is typical. For higher performance we move the filament forward towards the cap, resulting in a reduction in the filament life as it is worked harder to attaining higher emission/beam currents. The higher current enabling smaller spot sizes to be used whilst retaining a reasonable signal level. For longer filament life we move it back from the cathode cap, this reduces the emission/beam current, but will considerably increase filament life to the determent of image quality, just not enough electrons at the specimen level! Whilst I know many instruments that run up to 2,000 hours of filament life, they are not used for high quality imaging. However, many times in my career I have found the reason for poor performance is that the SEM laboratory is not pushing the filament hard enough; you have to have a reasonable emission/beam current (80-120 μA) if you want high performance. So if we look at Jason's problem, a short filament life would tend to suggest a high emission/beam current! Assuming sensible levels of saturation the other reason for poor filament life would be a poor vacuum in the gun, but a SEM running for 50 hours on a filament would not suggest poor vacuum. Moving on to his real problem, he is unable to obtain good quality images at 5kV because his stigmators are hard against the stop! When you work at low kV the cleanliness of the column is most important. At high kV the beam is able to penetrate column contamination to find an earth; no problems. Excess astigmatism will be the result if the kV is lowered, reducing its ability to penetrate column contamination; producing excess charge. So how does Jason overcome his problems? Firstly clean the final aperture, if this does not work clean the aperture holder, if this does not work, check the ESEM full alignment and still no solution, then finally clean the complete column. A piece of advice to help operators but unfortunately to upset some of my many service engineer friends. When a SEM is serviced a 30 kV picture will tell you that the gun is clean and happy, but more importantly in this era of low kV a low kV picture will show that the column is really clean. I have this saying that my dog could clean a column suitable for a good picture at 30 kV but it takes a good service to clean the column for 2 kV! Steve Chapman <protrain@emcourses.com> Feb 21

Scanning He-ion microscope

I wonder if someone of the list has already worked with this microscope. Has someone a feedback to share? What is the vacuum necessary in the column and specimen chamber? Is a low-vacuum possible? What are the qualities and drawbacks of this system? What about EDX analysis? Stephane Nizets <nizets2@yahoo.com> Apr 10

NETNOTES

I have not used one of these instruments yet, but I can easily answer one of your questions. XEDS analysis is not possible using the He Ion microscope. The reason for this is that the kinetic energy of the He ion beam is insufficient to ionize the inner shells of the respective atomic species. You will need an ion beam accelerated to MV not KV to generate characteristic x-rays. However, other types of analysis based upon the energy of the backscattered ion beam might be possible. Nestor Zaluzec <zaluzec@microscopy.com> Apr 10

How about something like Ion Backscattered Diffraction, a word swap equivalent to electron backscattered diffraction (EBSD). Is this a possibility? Is it useful?Michael M. Cheatham mmcheath@syr.edu Apr 10

An excellent article on this subject by R. Schwarzer can be found in the January issue of Microscopy Today, pg 34. The Acronym swap has "Crystal Orientation Maps", or COM replacing EBSD. Ron Anderson <randerson20@tampabay.rr.com> Apr 10

I am from the ALIS unit of Carl Zeiss SMT, the manufacturer of the Helium Ion Microscope (HIM). In response to your questions: The specimen chamber is held at a vacuum similar to what would be found in a FIB or high vacuum SEM: around 10^{-7} torr. The gun base pressure must be at near UHV (10^{-9} torr); however, it is much higher when running the ion gun due to the flow of the source gas to the emitter. There is differential pumping in the column to maintain the desired pressure in each region. Low vacuum is generally not possible in any ion beam microscope because the scattering cross section is much higher for an ion beam traversing a gas than for an electron beam. Above a pressure of about 10^{-4} torr significant spreading of the primary beam is noticeable. However, at least one of the two reasons that one typically pursues low vacuum operation is charge control, and this is accomplished in an ion beam tool by the use of a low energy electron flood gun. Sample hydration is the other driver for low vacuum, but there is no immediate solution for that application. Three notable features of HIM are the small probe size, the reduced interaction volume with the sample, and the different contrast mechanisms created by the signal generation from a primary helium ion beam. The high brightness of the source (at least 6E9 at voltage) allow for sub-nanometer probes to be formed. A probe size of 0.25 nm should be achievable, 0.5 nm has been measured. The low energy of the secondary electrons created by an impinging ion beam in a sample translates into a small escape depth (a few nm). Combined with the small lateral size of the probe, this means that the volume probed per pixel is small (no BSE's or SE-II's). Finally, the contrast mechanisms are qualitatively different for an ion beam generated image, giving different image information - and generally more gray levels than an SEM image. It is also possible to collect backscattered helium ions for imaging (since the ion mass is low), yielding a further type of analysis. To answer another question posed, there is an analogue for ions to EBSD: it is referred to as the ion blocking pattern, IBP. One can read about it in the January, 2008 article in Microscopy Today (p.34). Surfaces do need to be kept clean for the technique to work well - the microscope can both create and see hydrocarbon deposition better than an SEM. This can be addressed through engineering, however, and is not a fundamental limitation. Sputtering damage is minimal, but not zero. Helium implantation damage can occur, but this is

https://doi.org/10.1017/S1551929500059319 Published online by Cambridge University Press

usually at a dose much higher than is required for imaging. Nestor already answered correctly about EDX. However, the energy analysis of backscattered helium ions can provide elemental analysis akin to RBS. We are developing this capability. Larry Scipioni <l.scipioni@smt.zeiss.com> Apr 15

EDS - accuracy of thin film measurements

We are a small company making phase change memories. We sputter deposit chalcogenide materials with the thickness of the films ranging from 5-75 nm. We have an FEI NNL dual beam system with an EDS, and a WDS systems on board. We use this tool to check for thickness and composition (using EDS) of the films we deposit. We cleave the wafer and look at it edge on for accurate thickness measurements. Our questions are as follows: 1. Using about 5-20 KeV and about 1 nm spot size, how accurate can we get the composition of the films that we deposit, would 10% be reasonable and attainable? That is our challenge currently. We can get a reasonable cps and dead time in our EDS system. 2. Any other method of getting accurate composition measurements on these films? Bobby Hooghan <hooghan@verizon.net> Mar 24

The bottom line is that if one does not correct for the thin film geometry one will have unpredictably poor accuracy whenever substrate x-rays are observed. If you are interested in details, please contact me off-line and I can send you some PPT slides that demonstrate this with a NIST thin film standard I measured. John Donovan <donovan@uoregon.edu> Mar 24

EDS - analysis on thin contaminant layer

Anyone out there has experience with EDS on aluminum bond pads? Sometimes due to certain wafer fabrication process, a thin fluorocarbon contamination layer is formed on the pads. I notice that if I use low kV (3-5kV) and tilt the sample (60 degrees or more) I get an increase in the sensitivity of detecting the fluorocarbon layer compared to the case of zero tilt. I assume by tilting the sample the interaction volume is shallower and closer to the surface. My question: is sample tilting in such circumstances acceptable and would it cause any quantification errors? WF Kho <wfkho@streamyx.com> Mar 12

Tilting the die will yield more volumetric interaction with the bond pad metal. How much depends on your KV. F is pretty easy to detect and discriminate since it only has a K alpha peak at 0.677 eV. So the challenge is to determine what other elements you want to include in a quant. 4KV-5 KV would work well for F. If you are going to include Al and Si or lighter elements, perhaps 5KV-6 KV would do a nice job--these elements would also be using the K alpha peaks. If the KV is too low, you will see this as high intensity ratio error. That would mean to up the KV a little until the ratio came down. Less than 10% or so is a decent value. When you tilt, what happens to cps and DT? Is WD the same? Gary Gaugler <gary@gaugler.com> Mar 12

Quantifying this system is an exceedingly difficult problem and realistically one, which should not be attempted. There are many reasons. Here are a few. 1. It is possible to analyze thin films on top of known materials but you must use a thin-film-type quantitative correction algorithm. Your EDS software might provide one. 2. It is possible to analyze tilted samples but the correction algorithm must correct for the different absorption lengths due to the oblique incidence and the foreshortened exit path. In addition, the backscatter yield will be influenced by the oblique angle. Your EDS software might provide a tilted sample correction but I'd be skeptical about the results. Quantitative correction algorithms work best the closer your sample is to a flat, polished bulk sample at normal incidence. At a moderate cost, you may get away with a single deviation from this ideal but two or more deviations - forget about it. 3. Fluorocarbons contain at a minimum F, C, O and H. They may also contain Br and Cl. Since there are no H emission lines, we must estimate H by difference. The remaining elements are low Z and thus low energy. The line intensities will all be strongly modified by absorption. Strong absorption will further complicate issues 1 and 2. At normal incidence on a bulk sample quantifying fluorocarbons is a challenge, at oblique incidence on a thin film, quantification is nearly impossible. In a system like this, the best you can realistically expect is qualitative results. You can answer questions like is there any fluorine? Oxygen? Carbon? Bromine? You might even be able to make major/minor/trace type distinctions. Your EDS software may happily spit out quantitative results to many decimal places. Don't believe any of it. You shouldn't even trust the first decimal digit. Nicholas Ritchie <nicholas.ritchie@nist.gov> Mar 12

EDS - effect of beam alignment on results

I have received a request from a user who wants to have an image of the SEM emission image along with EDS results. The user claims that he wants to use that information to estimate error in EDS analysis. This is the first time I have heard something like this. What would be the effect of e-beam alignment on EDS results, as long as it is aligned well (best we can do manually) along the optic axis? Ayten Celik-Aktas <celikaktas@gmail.com> Apr 12

Which error is he talking about? If he is referring to spatial resolution, this is largely (entirely?) dependent on the sample. Larry Stoter <larry@cymru666.plus.com> Apr 12

I can readily show you BSE images (and x-ray maps) of polished samples taken at low magnification that show brightness variation across the field. There is a substantial fall-off in brightness toward the corners. Presumably, the bright area is centered in the field. If not then there is need for alignment. However, I am afraid that this client is straining at a gnat and swallowing a camel. Are the EDS analyses being done without normalizing the results? If so, the brightness variation will affect the totals or any element determined by difference. I expect the results are being normalized so there would be no effect. Does your client understand the distinction? With normalization, a phase probed at the corner of the field would report the same composition as the same phase probed at the center of the field. Is the spectrum being collected from a homogeneous area? Perhaps the user wants to be sure that all phases are being represented evenly across the field of view. That is understandable - but wrong! EDS matrix corrections assume a homogenous sample volume. Let's assume a Ni-Al sample prepared by butting up chunks of the two elements against each other. If you are correcting the Al emission from one side for the effect of Ni atoms on the other side, that would be wrong. The generation of x-rays and their interaction with both Al and Ni atoms would only occur along a narrow band of the interface between the two phases. Otherwise, the spectrum is just the sum of the signals from the pure phases. Virtually no correction would be necessary. Is the client aware of the accuracy limitations of EDS? If they are inter-comparing analyses taken on the same system, they may be able to make some sensitive com-

parisons. However, counting statistics probably limit repeatability to a few tenths of a percent or worse. Absolute accuracy could be off by a few percent. What level of improvement will result from their exercise? I maintain that EDS is quite a powerful technique - when done correctly. My impression is that the effect your client is concerned about is probably the least of their worries. Warren Straszheim <wesaia@iastate.edu> Apr 12

This client wants the screen capture image of the "electron beam" (how well the e-beam is centered, aligned) that we can see during alignment process. I'm not sure if this could be used for estimation of error for EDS results at all. Of course, I obtain SEM and BSE images of the areas that I have examine for EDS work. Ayten Celik-Aktas <celikaktas@gmail.com> Apr 13

That makes it clearer. I could not see how the alignment would have any effect on EDS accuracy. Maybe it does. It would affect probe current and volumetric interaction most likely. If it affects accuracy, how so? Depending on the SEM, getting a beam image can be easy. With LEO/Zeiss, use Emission mode. With FEI/Philips, use X-Over mode. Not informed about other brands. Gary Gaugler <gary@gaugler.com> Apr 13

I will second Warren's assessment of the situation. It sounds like your client is concerned about the molehills but is overlooking the mountains. Perhaps your client knows something about a different analytical technique (such as WDS, where beam alignment is important to preserve the Rowland circle geometry) and is trying to bring that bit of knowledge to EDS. Perhaps your client is simply working from specifications written by someone else and is holding to them because they are required to keep their procedures the same over time. Perhaps some earlier EDS analyst included this in a report, and now your client believes it is always important to know. Perhaps they're trying to "break the rules" of EDS, as Warren described, and are hoping a heterogeneous region will be evenly illuminated by the beam. There are many possibilities about why they want this information, but that's not the most important question right now. The question at this point, Ayten, is: What would you like us to help you do? I see two basic options: 1) Do you want ideas about a way to image your beam alignment and simply satisfy their request? 2) Do you want to educate them and convince them that this really isn't the biggest source of error? Option #1 is clearly the easiest one: you can satisfy your client and simply be finished with it. If I was asked to do this, I would either: (A) place a cathodoluminescent mineral in the microprobe, align the luminous spot with the cross-hairs of the visible-light microscope that is aligned with our electron optical column, capture an image of this, and give that image to the client; or (B) burn a hole in or leave a carbon deposition spot on a sample, capture an image of how the mark sits at the center of the electron image, and give that image to the client. Those methods aren't perfect, but I think that they would satisfy most clients who'd ask to know such a thing. Option #2 is probably the more "responsible" thing to do, but it is also going to be more difficult. I've been in a similar situation before. I've had to deal with a corporate client who wanted superalloy samples analyzed, and they wanted to start

https://doi.org/10.1017/S1551929500059319 Published online by Cambridge University Press

using my lab, rather than the lab they had been using, because my lab was within courier distance. The first step, though, was to show that I could give the same results as the other lab, and I had to use the procedures established by that other lab. The problem was that the other lab was using procedures with which I didn't agree, including rastering the beam across a heterogeneous area to get an "average" composition. This heterogeneous area included metals with serious issues of absorption and fluorescence, so the correction procedures were essentially being lied to. I voiced my concerns with the client, but they didn't get it. Nor did they understand that slightly different ZAF methods produced different results because of these ill-chosen procedures. In the end, the client didn't end up using our lab because consistency, not a better procedure, was more important to them. Educating a new corporate client about their misconceptions can be highly challenging, and sometimes they really don't want to know. Option #2 is the better for all of us as it would improve your client's understanding of X-ray microanalysis. But I certainly wouldn't blame you at all you for following Option #1 and just trying to give the client what they want (or think they want). Or you could try to do both Options #1 and #2 -- give them what they want and educate them why it is not important. Ellery E. Frahm <frah0010@umn.edu> Sun Apr 13

SILICON DRIFT DETECTORS - element mapping

Silicon drift detectors (SDDs) and pulse processors are really coming on strong. The newest generations of these are impressive. There is an issue I think with SDDs that has not been discussed. Or, I am missing something? A Si(Li) or SDD will do a nice job of collecting spectra of elements with good resolution (~129eV) when cps and DT are low with longest filter time. Not a problem. However, the resolution of EDS degrades as filter time is decreased. This is true for both types of detectors. The advertised ability of SDDs to collect at high cps is true if the filter time is reduced to keep DT low. So, suppose one is collecting a string of light elements--C, N, O, F, Na, Al and Si. Either detector can do this, if at highest resolution-- meaning, longest filter time. Now suppose the task is to do a fast map of these elements. Increasing cps via normal means causes DT to increase and thus requires shorter filter time. As this happens, resolution degrades to a point at some higher cps where the peaks are not individually resolved. The result is a useless map. Thus, the claim of high cps ability is true on one hand but not across the board for spectra and mapping. Yes? For Si(Li) and it seems for SDD mapping, one must create two element lists such that there is sufficient eV resolution between collected elements to result in meaningful maps. An example might be C, O, Na and Si as list one. List two would then be N, F, Al. It might be necessary to have three lists so that one could take advantage of higher cps and shorter mapping time. This would also help in drift correction. Perhaps there are other methods or details I am missing? Gary Gaugler <gary@gaugler.com> Feb 15

Not quite. SDDs routinely get good resolution with processing times around 1/10 to 1/20 of the Si(Li) processing times required for best resolution. So depending on your definition of "high", it is perfectly reasonable to run the same processing time always with an SDD. You can get to 30kcps easily, which would be screaming with a Si(Li), and keep the resolution around 130 eV. In many cases, you won't generate more than that if you want to keep the spatial resolution good. Yes, this depends on the particular SDD/processor combo you have. They aren't all the same, and there have been generation changes just in the last year or so. Anything in the mid-130's is good enough to separate C, N, O and F nicely. Low 130's to mid 120's even splits the L_l lines from the L-alphas for the transition metals, which is awesome but probably not required for mapping. There's another key point, which relates to Stephane's query of a few days ago (to which I will reply separately). The dead time will be much lower with SDDs than with Si(Li)'s, again because of the shorter processing time, which means you collect more of the X-rays you generate. At 30kcps and 130 eV or so, you can stay down around 10-15% dead time. That's a big win. Pile-up (sum peaks) matters for the list of elements you describe as the rates go up, and their relative intensities are proportional to the *input* count rate, not throughput rate. 100 kcps at 50% DT is six times the input rate of 30kcps at 10% DT, so all other things being equal, the sum peaks will be 6x bigger in relative terms, not 3x. The O sum peak is within 9 eV of the Na K, for example. So it's a bit risky to try for the highest rates touted for the SDD, if you have significant peaks below 1 keV in the spectrum. If you're only mapping major elements, you can mostly ignore the pile-up, but if you want to pick up minor elements it can get you in trouble, especially if you want to try anything resembling "quantitative mapping". Rick Mott <rmott@pulsetor.com> Feb 15

INSTRUMENTATION – diffusion pump

What's the most practicable solvent for cleaning old (but not burnt) Santovac from a diffusion pump? Ritchie Sims <r.sims@auckland.ac.nz> Apr 6

We use Solvon B or acetone here at Ladd. Mike Bouchard <sales@laddresearch.com> Apr 7

The Santovac diffusion pump fluid is a polyphenyl ether compound with a molecular weight of about 455. As such, it is both highly viscous and rather insoluble. (see p.183-4 of Vacuum Methods in Electron Microscopy). However, since it is based on a molecular structure consisting of benzene rings (i.e. the phenyl groups) it will tend to be most soluble in solvents that also contain benzene rings, such as toluene and xylene. Acetone and methyl-ethyl-ketone are also helpful solvents, and mixtures of these with toluene and xylene are sometimes recommended. The usual procedure is to wipe off as much of the fluid as possible with dry rags or tissues, then follow up by wiping with pads moistened with one of these solvents. For parts that can withstand such mistreatment I have also found that washing with hot water and liberal amounts of one of the modern detergents that are formulated for degreasing automobile engines can be helpful. Finally, rinse with isopropyl alcohol. Wilbur C. Bigelow <bigelow@umich.edu> Apr 7

Many thanks to all who responded to my query, in fact I found trichloroethylene (1,1,2-trichloroethene) to be more effective than either toluene or isopropanol for dissolving the Santovac that was left on the DP parts after I had heated them to 60 deg and wiped them with tissues. I imagine that the more-widely-available perchloroethylene (tetrachloroethene) would work just as well. Then I rinsed with isopropanol and dried in the 60° oven before reassembly. Ritchie Sims <r.sims@auckland.ac.nz> Apr 10