# NETNOTES

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**Selected postings from the Microscopy Listserver from 10/16/08 to 12/15/08. Complete listings and subscription information can be obtained at http://www.microscopy.com. Postings may have been edited to conserve space or for clarity.**

## SPECIMEN PREPARATION - glutaraldehyde shelf life

*I just found 20 sealed ampoules of 10ml 50% EM Grade glutaraldehyde. Stored in a refrigerator, purchase from one of the EM supply companies. But they were purchased in 1994. Yes, we could yes go ahead and prep some samples, but rather than waste the time and effort I thought I'd get the feeling from all you folks: (1) Garbage?, (2) Likely to be good?, (3) I don't know, do I have to test? Richard E. Edelmann edelmare@muohio.edu*

I don't know the glutaraldehyde shelf life but am interested since I have some 2-3 year old ampoules myself. I am guessing it is good. I can't resist mentioning that two years ago I got a huge stock of over 20 grams of free crystalline osmium tetroxide - the only problem was that it was packaged by Merck in 1940! The ampoules were in individual wooden sleeves. I found no difference in osmication with it compared to recently bought osmium. Tom Phillips phillipst@missouri.edu Thu Dec 11

This nudged my memory. Take a look in Hayat, Principles and Techniques, Vol. 1, pp. 78-81 You might be able to scan it on a UV spectrophotometer. Pure glut absorbs at 280 nm, other peaks would indicate impurities. Glen MacDonald glenmac@u.washington.edu Thu Dec 11

We prefer to use fresh glutaraldehyde - usually 1 year old maximum but sometimes it's a bit older - I always ask the person fixing the tissue if they care or not. Some older faculty actually preferred really old glutaraldehyde for their marine samples - not sure why. And, I agree, It seems the age of the osmium doesn't ever seem to matter - it's always good. Beth Richardson beth@plantbio.uga.edu Thu Dec 11

I would not use 3 year-old glutaraldehyde. I had a sad experience of doing perfusion-fixations using fixative containing 3 year-old 50% glut from a respected EM vendor, in sealed amber ampoules, stored at-20°C (it does not freeze at this temp). The fixative solution was formulated properly and tested correctly for pH and osmolarity. The animals had been on a 6 or 9 week protocol- thus expensive animals. The perfusion was excellent, judged by the open lumens in the kidneys, but the cellular fixation was terrible. Unusable. The glutaraldehyde had no expiration date on it, but we called the vendor and they told us at the time that the shelf life was approximately 14-16 months. Now I think they are saying 2 years shelf life. My advice is: Don't take a chance and don't waste your time and resources testing it. Glutaraldehyde is cheap. Labor, animals, time, other supplies are very expensive. Jill Verlander Reed Jill.Verlander@medicine.ufl.edu Thu Dec 11

## SPECIMEN PREPARATION – Spurr's resin

*I have been using toluidine blue to stain "thick" sections (1 µm) of embedded brain tissue in Spurr's resin. I am not happy with the quality of the histology/stain. Can someone suggest a better stain/method for material embedded in Spurr's? Susan C. Van Horn susan.vanhorn@sunysb.edu Mon Oct 20*

Have you tried adding 1% sodium tetraborate (borax) to the toluidine blue? You also need to heat the section while staining. This works for me, although I mainly work on plant tissue and I cannot vouch for its efficacy with the new Spurr formulations. Carol Evered carol.evered@warwick.ac.uk Mon Oct 20

There are a great many papers published on staining "thick" sections. The ones that give good results, at least in the hands of the authors, are long and very complicated. While slides stained with such methods look very nice, they still are not as pretty as a proper hematoxylin and eosin. Thus, the popularity of toluidine blue in sodium metaborate, simple and effective. If you feel that you must try something better I will send you a partial listing of such "improved" methods. Geoff McAuliffe mcauliff@umdnj.edu Mon Oct 20

## SPECIMEN PREPARATION – processing paraffin specimens for TEM

*In response to a question on processing paraffin embedded tissues for TEM, the following responses were made:*

We process paraffin embedded tissue more often than we would like. You are correct, the results are far from optimum, probably less so than if were left in formalin! Our pathologist can sometimes make a diagnosis from the images but I doubt if they would ever publish them. If that is all the tissue you have, it may be worth a shot to give it a try. We cut out the area of the tissue desired; keeping as little paraffin around the tissue as possible then put the tissue piece into xylene overnight to melt/dissolve the paraffin. We then rehydrate from 100% ETOH to buffer, fix for at least 1 hour in Karnovsky's (our routine EM fix) then process as we do our other clinical samples. Years ago I worked for a clinical EM lab where we freshly mixed Osmium tetroxide with toluene and used that for the secondary fix. We didn't rehydrate the tissue just went on to embedding. Sorry, I don't have that protocol but maybe someone else can help you with that one. Good luck. Skin is tough so maybe you can get what your researcher needs. Pat Kysar pekysar@ucdavis.edu Wed Nov 19

Yes the ultrastructure will be terrible, but may be adequate for an answer. I dissolve $OsO_4$ crystals in toluene (1%), cut out the tissue from the paraffin block, mince into 1 mm pieces and place pieces in the osmium/toluene overnight. Rinse with acetone three times to remove osmium and then infiltrate as you normally would with your standard epoxy resin. The paraffin processing will remove most membranes, all lipids, shrink the tissue about 20%, and numerous other artifacts will appear. Exactly what type of cells are they looking for? Cytoplasmic filaments will be stable. Most protein inclusions will stay put but the ultrastructure may be hard to recognize. Cell junctions will still be present. The main thing to let the person know is that the results may not yield any valid answer and get them to think of EM to begin with. Edward P. Calomeni edward.calomeni@osumc.edu Thu Nov 20

## MICROTOMY – flattening sections

*In biological specimen preparation for TEM, one uses chloroform to straighten out sections (on the surface of the water bath in front of the knife). Chloroform is also used as the solvent for pioloform, one of the plastic films used to coat slot grids. Does anyone know of*

*something that would work like chloroform but which wouldn't be as poisonous to humans? Does anyone have a solution for how not to end up poisoned by chloroform? Open windows, gas masks, ventilation etc don't work - try cutting 50nm serial sections in a breeze and see what happens. Every time I come into contact with chloroform I end up with a full-blown migraine. As these tend to last about 5 days, it's a bit of a problem when I'm sectioning every day or every other day, and when my career largely depends on my skills as an electron microscopist. Giselle Walker gw265@cam.ac.uk Tue Oct 28*

You really shouldn't be using chloroform in the open any more, it's just too hazardous. A risk and COSHH assessment should have been carried out in any case. Flattening of sections is easy to answer - you just use a small portable heat pen and gently waft it just above your sections. These should be available from companies such as Agar Scientific and have been a standard piece of kit for many years in my labs. I still use chloroform as a solvent for plastic films but I simply segregate all of the solvent handling procedures into the fume hood. I dip glass slides in a measuring cylinder containing plastic in chloroform and store them in a Petri dish standing up against a small beaker and covered by a larger glass beaker while the chloroform evaporates. This can all be done in the fume hood. After about 5 minutes I remove the Petri dish and beakers and cast my dry films on water in the open lab. I hope this helps. But you must not expose yourself to chloroform it carries a fair list of hazards including: R20 Harmful by inhalation. R22 Harmful if swallowed. R38 Irritating to skin. R40 Possible risk of irreversible effects. R48 Danger of serious damage to health by prolonged exposure. It is also regarded as a potential carcinogen. You must have a fume hood somewhere nearby to handle glutaraldehyde, osmium and harmful resins anyway. I hope this helps, but please stop using chloroform in the open and you should seek advice from Occupational Health about your past exposure. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Tue Oct 28

This is a good question to get the controversies stirred up because if the biological specimen is properly infiltrated with resin, and the resin has been correctly mixed, then you should not need to spread the sections after they have been cut - ever! I have not used chloroform for years, purchased a battery-operated heat pen that lost its heating ability within the first weeks of use, and then had a long conversation with Hildy Crowley about embedding. She was correct in stating that full infiltration stops section compression sufficiently to make flattening unnecessary. For really good infiltration I leave specimens for up to two weeks in uncatalyzed resin (the really difficult cells are yeast with cell walls still attached), before transferring them to resin with catalyst. Works for me. Paul Webster pwebster@hei.org Tue Oct 28

My resin blocks are all very hard, especially compared with some of the old specimens I found in the drawers when I arrived here. I know that soft blocks work best for glass knives but we have the luxury of being able to use diamonds for almost all our sectioning (even semi-thins). With the yeast, we have really big problems processing and embedding the unspheroplasted cells (not taking off the cell wall) even using the methods used by the yeast experts. We devised a way of embedding that worked for us and has been reproducible; however, we now have a high pressure freezer that is an idea way of "fixing" yeast, so we don't use this method any more. Basically, the yeast are chemically fixed (formaldehyde and glutaraldehyde). Usually the cells are mailed to me after fixation, so they arrive as a suspension. They are then washed with buffer containing lysine to quench the aldehyde and embedded in either 2% gelatin or agarose. I prefer the gelatin because it is easier to step back if the cells don't centrifuge well. The blocks are re-fixed in aldehyde to fix the gelatin (agarose) and sliced into really thin pieces. Too many yeast cells aggregating together will never infiltrate, so the thinner the slices are, the better the embedding. The gelatin fixation and subsequent osmication, dehydration through ethanol or acetone, and first infiltration steps are performed in a microwave processor, and rushed through. Typically we get to 1:1 resin:solvent in less than 3 hr. The blocks are then transferred to 1:3 (more resin, still no catalyst) and left overnight. They are then transferred to 100% resin (no catalyst) and left on a rocking machine for many days. After about a week, some blocks are taken out, mixed with fresh resin, with catalyst (BDMA), left for about 30 min, transferred to fresh, catalyzed resin and baked in a 60°C oven. If the blocks are not well infiltrated, we go back to the specimens in the resin and embed a few more, until we get sections that can be used in the TEM. Usually we can get edges of some blocks to section sufficiently well after a few days infiltration. However, the better blocks appear after a couple of weeks. This has all been worked out by trial and error using the very plentiful supply of yeast cultures sent to me by other people. Sometimes they did have to wait a very long time to get a result, but I did warn them in advance that they could get a more rapid result if they sent someone to do the work. I don't charge for this work so can decide to do the work on not, so the end result is on my time, not that of a paying customer. I did some preliminary experiments with the specimens infiltrated with uncatalyzed resin, because, like Gib, I was curious how the catalyst could affect tissues already filled with uncatalyzed resin. Even with no pre-soak in fresh catalyzed resin, many of the blocks become very hard after baking, suggesting that the catalyst is able to affect the resin already in the cells. Remember, my tip about cutting the blocks into very thin slices. I think that is the secret to how this works. The morphology of these cells is never as good as those that have had the cell wall removed, and are not nearly as good as the high pressure frozen, freeze substituted cells. Paul Webster pwebster@hei.org Tue Oct 28

Anyone who works with plant material with even one cell layer with impermeable cell walls is used to long infiltration times. Once had a student working on resurrection plants, and infiltration of the dry material was up to 8 weeks, and even longer was better. If you want to avoid artifacts like membrane and cell wall breakage, give serious consideration to the drying down process in these plants, a topic often discussed at length, but really this is just an artifact of poor fixation and infiltration. We routinely take 2 months to process dry seeds. There are lots of other artifacts from too fast infiltration as well, even in tiny Arabidopsis roots, the mature endodermis can block infiltration and result in cytorrhysis. Someone said to me that "roots always look like this".... well, no they don't, not if you take the time to do it properly! These days, we try to avoid this by working on fresh or at least non-embedded material, but sometimes have to go to embedding. Rosemary White <rosemary.W=white@csiro.au>| Tue Oct 28

Referring back to the waving-chloroform-over-thin-sections thread, there were comments about using heat pens (mea culpa) and how they eat batteries. There is a simple solution to this problem: a 6 V microscopy-lamp power supply. The kind used with separate lights on stereoscopes. Usually there are a few extras lurking in drawers and cabinets from old microscopes. Take about a meter (+/- by need) of lamp cord. On one end wire on the appropriate plu for the power supply, then at the other end, strip bare and solder the wires to the battery connectors in the heat pen. No more batteries needed, and you now have a variable-temperature (sort of) heat pen. We usually don't need more than 4V on the power supply selector, that's plenty enough to burn fingers or spread sections. This was originally done by my predecessor here, Geoff Williams. Philip Oshel oshel1pe@cmich.edu Fri Oct 31

We use an electric heat pen with great luck on Epon thin sections but had only modest success with 0.5-1.0 μ thick sections. It does slightly better for butyl, methyl-methacrylate sections. I have had no luck with the battery powered versions. I probably will try the design of Geoff Williams that Phil described just for kicks. I knew I saved all those all lamps for something. Tom Phillips phillipst@missouri.edu Mon Nov 3

I would normally warm up thick or semi-thick sections (~1 μm and thicker) on a drop of water on a glass slide using a hot plate or slide warming plate. You can leave the sections for much longer to spread out. Perhaps not so easy if the sections are on a grid - but possible I'm sure. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Mon Nov 3

The way I use our heat pen is to pick up the sections (floating on a large water droplet) on naked grids and pass the grid between the heated loops of our heat pen. Our heat pen does not get as hot as yours, so there is no danger of vaporizing the water/sections as with your pen. However, you might try picking up sections so that they are floating on a large droplet on the grid and slowly approach your pen until you see them relax. Observe the relaxation under a stereomicroscope, if possible. Also, do this using expendible sections, until you figure out if this will work reliably for you. If you try to relax the sections as they float in the water trough of the knife, they will run away from the heat. You need to confine them (either on the grid, or a loop containing the sections). If you have problems with the grid method, then use a wire loop (like the EMS Perfect Loop) to warm the sections and then bring down the loop over your grids so the sections transfer. Let us know how you make out. John J. Bozzola bozzola@siu.edu Thu Dec 4

I have had the same experience that you describe. I have a homemade heat pen and don't find it very effective when held over the sections in the knife trough; and I get the same wild section movements and almost no flattening. But there is a picture of sections in a drop of water held on a grid that is placed through





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the center of a heater hairpin loop - this is in the Bozzola and Russell book (Electron Microscopy - Principles and Techniques for Biologists (Fig 4-44b of 1992 1st edition)); this is a different style of heater loop than you have purchased; it is longer and more open. I have not yet tried this because I don't pick up sections that way in general, but wonder if it wouldn't be more effective since they get heated from both sides in a much smaller volume of water; I think that the sections on the trough surface are never very hot due to the large volume of water below and all the motion. Dale Callaham dac@research.umass.edu Thu Dec 4

I suppose I should make comment, as I did to your first enquiry. I have used Spurr's for many years and had few problems with flattening silver/gold (80-100nm) sections on an ultramicrotome cutting at 1-2mm/sec speed. I have always used a medium/hard mix of Spurr's with few problems unless the embedding has been soft. I can think of several possible sources of the problem: The heat pen that I use is a tungsten wire type which glows red/yellow but certainly not white. Mine uses a fixed mains electric controller, but some used to be available with a variable heat adjustment. Another possible source of problems is the change of formulation of Spurr's resin over the last year or so. It would be worth you trying some much older Spurr's blocks if you are using the new Spurr's. A final issue that sometimes arises in some environments is the build up of static on the surface of sections which tends to make the sections disperse when you approach with a heat pen or tweezers and grid sometimes. Humidifiers or anti-static guns may solve this but it would be important to establish that this is the problem before spending more money. I hope this is of some help and will keep you away from the dreaded chloroform. Malcolm Haswell malcolm.haswell@sunderland.ac.uk Fri Dec 5

MSDS chloroform: Warning! May cause severe eye irritation and possible injury. Flammable liquid and vapor. May be absorbed through intact skin. May cause skin and respiratory tract irritation. May cause central nervous system depression. May cause liver damage. May cause kidney damage. May cause adverse reproductive effects. Target Organs: Kidneys, central nervous system, liver. Potential Health Effects Eye: Contact with eyes may cause severe irritation, and possible eye burns. Skin: May cause skin irritation. Prolonged and/or repeated contact may cause defatting of the skin and dermatitis. May be absorbed through the skin. Ingestion: Cannot be made non-poisonous. Causes gastrointestinal irritation with nausea, vomiting and diarrhea. May cause kidney damage. May cause liver damage. May cause central nervous system depression, characterized by excitement, followed by headache, dizziness, drowsiness, and nausea. Advanced stages may cause collapse, unconsciousness, coma and possible death due to respiratory failure. Inhalation: Inhalation of high concentrations may cause central nervous system effects characterized by nausea, headache, dizziness, unconsciousness and coma. May cause respiratory tract irritation. Prolonged exposure may result in dizziness and general weakness. Chronic: Prolonged or repeated eye contact may cause conjunctivitis. Prolonged or repeated exposure may cause adverse reproductive effects. May cause liver and kidney damage. Section 4 - First Aid Measures Eyes: Immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical aid. Skin: Flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid if irritation develops or persists. Remove contaminated clothing and shoes. ... Very interesting reading, isn't it? But it is not about chloroform, it is about very pure ethyl alcohol-D, 99.5+ Atom % D. In this MSDS I like especially part about removing clothes in case of contamination. Shouldn't it be posted in all bars? Then, if you splash whiskey on your trousers you should remove them immediately, otherwise you will be transported to physician at your own expense. Vladimir M. Dusevich dusevichv@umkc.edu Wed Dec 10

Not to throw a whole other monkey wrench into the mix of heat pen vs solvent... For Spurr's and Moll/Epons, I've only ever used xylenes to relax the sections. I still have the "wand" I made as an undergraduate, and still use it. A small corner of filter paper attached to the end of a wood applicator stick. I mostly use Spurr's and find the xylenes work great. That or I just walk away (or get up to answer questions or help someone else) and they are nice and relaxed when I come back to sit down again. The heat pen mentioned earlier on the list is a jeweler's model, and I always had great success stretching Spurr's sections with it. It does take a touch more time than the xylenes and it does work better on thinner rather than thicker sections. Are xylenes better or worse for you than chloroform? Honestly off the top of my head they are both in the nasty chemical category and I don't have a least favorite, just habit I guess. I will likely be moving the lab over to heat pens soon, but I will not prevent users of our facility from using their method of choice to relax sections. I just wanted to chime in on a chloroform alternative. Geoff Williams geoffrey_williams@brown.edu Wed Dec 10

**MICROTOMY – wetting the knife**

*I am desperately trying to wet my diamond knife (Diatome) both soaking it in detergent or wiping with Styropor and alcohol to no avail. Would you have a miraculous method to wet your knife? Any suggestions? Stephane Nizets nizets2@yahoo.com Tue Nov 11*

Here is my most elegant solution: Spit on your finger. Take your eyelash stick (i.e., an eyelash attached to a thin wooden stick) and drag it through the protein-rich saliva. Gently wipe this on the downslope of the diamond knife edge. Viola! Thomas E. Phillips phillipst@missouri.edu Tue Nov 11

That's a trick that I've been using for years....suggested to me by the wizards at Diatome. It does work beautifully, even if its got a high "yuck" factor. Lee Cohen-Gould lcgould@med.cornell.edu Tue Nov 11

Put it in a plasma, glow discharge. Should make it nicely hydrophillic. Tobias I. Baskin baskin@bio.umass.edu Tue Nov 11

**MICROTOMY - coated grids**

*Has anyone used Luxel's film-coated grids? http://www.luxel.com/ The website says the film was developed with the help of NIH. Are they great? or not so great? Thanks for your comments, Beth Richardson beth@plantbio.uga.edu Tue Dec 2*

We were given some to try and they worked very well. Our users liked the increase in open viewing space. One thing we did find was that LR White sections did not adhere to them but epoxy ones were just fine. Lesley Bechtold lesley.bechtold@jax.org Tue Dec 2

# NETNOTES

**IMMUNOCYTOCHEMISTRY - fluorescence quenching**

*I labeled a substance with an Alexa488 dye. Now I would like to see its internalization in cells in culture. My main issue is that I have no confocal microscope, so I have to find a way to eliminate the extracellular fluorescence and keep only the fluorescence inside the cells. I know that it is possible to quench the fluorescence with Trypan blue, but I don't understand exactly how it works. Does Trypan blue stick to the dye and quench it, so that even after washing the fluorescence is still quenched? Or do you have to leave the Trypan blue is solution (I wonder how it can influence imaging)? Does someone have instructions to do that? How long, how diluted? Is the pH important/critical? How long is the quenching stable? Stephane Nizets nizets2@yahoo.com Wed Dec 3*

There are several possible ways that fluorescence can be quenched, and I do not know how Trypan blue works. Some compounds provide a non-radiative pathway for decay of the fluorophor from the excited state to the ground state. This can be either by binding or by being near enough to have a high probability of energy transfer--think FRET without the shifted photon. Other compounds change the environment, especially hydrophobicity or hydrophilicity, which only work when fluorophors require a specific environment to fluoresce. Some fluorophors, for example, emit only when intercalated into membranes or DNA, etc. The details of how to continue to have Alexa488 continue to fluoresce within the cells, but not have other intracellular fluorescence and how Alexa488 interacts with Trypan blue, I'll leave to the experts. Washing out Alexa488 that is not within your cells should be pretty straightforward and will eliminate extracellular fluorescence, but, again, take an expert's opinion over mine. Bill Tivol tivol@caltech.edu Wed Dec 3

I have been reading the thread on fluorescence quenching with interest, and wonder if anyone can give a protocol for using Trypan blue. All the books say it is minimally soluble in water. Shea Miller millers@agr.gc.ca Wed Dec 3

Generally, it is auto-fluorescence in the cells or tissue that you wish to quench - you normally don't have loads of fluorochrome fluorescing in the culture/mounting media as such. You replace the media prior to imaging, remove it during washes, and say use a clear $CO_2$ independent culture media for short time-lapse of live cells rather than use the standard red tinted culture media stuff that happily autofluoresces a bit. We used microscope flow cells and micro-injectors back at UCL, and I don't remember getting serious background fluorescence problems. I have used the excellent Wright Cell Imaging Facility's article on autofluorescence many times and it's linked here along with some other useful autofluorescence related stuff]. It discusses your problem. http://www.cbm.uam.es/confocal/Ingles/autofluorescence.htm Except the main link doesn't work, so get it direct from: http://www.uhnresearch.ca/facilities/wcif/PDF/Autofluorescence.pdf. And just search 'auto fluorescence', 'autofluorescence', 'auto-fluorescence', and 'reducing', 'quenching', 'eliminating' and so on..... Although it's not 'autofluorescence' you are trying to remove, the info will still be relevant - some of it might be aimed at quenching autofluorescence inside the cell though. Note: Amongst other things, Trypan blue is used to quench auto-fluorescence - never actually tried it as generally, say with eye sections/cells, we use the autofluorescence to our advantage and actually don't want



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it to fade. With a confocal you can just about image anything that is or has lived using autofluorescence; it's just that generally the autofluorescence brightness is well below the fluorochrome you have added, so you never see it. You simply image unstained cells/tissue as well to check whether autofluorescence was a problem at the imaging gain/laser power or camera exposure you use with the fluorochrome sample. Some cells are easy to identify owing to their bright autofluorescence; e.g., autofluorescence in our eye's rods and cones can be used to detect the early onset of eye disease, etc. Keith J. Morris kjmorris@well.ox.ac.uk Thu Dec 4

**IMAGE PROCESSING - reference image subtraction**

*I have some "dirt" in my images that I assume is due to a dirty camera chip. I've cleaned everything I can in the optical path, Kohlered the illumination, and I still have these "spots" (they're more like regions of darker shading) in every single image. I know there has to be a way to "Subtract" a reference image of this stuff (taken away from my sample) from my sample images. I have Photoshop CS. Can anyone tell me how to do this?? Danielle Crippen Wed dcrippen@buckinstitute.org Wed Nov 12*

Normal disclaimers aside, try Photoshop rubber stamp. If the anomalies are small, this should do the trick. For more industrial strength applications, I'd use Fovea Pro. Gary Gaugler gary@gaugler.com Wed Nov 12

Whatever you do, remember that you need to report it as part of your image acquisition or post-acquisition manipulation! Reference image subtraction is preferable to using any Photoshop filter or tool (except brightness, contrast, and histogram stretching). The rubber stamp is especially nasty as it fundamentally alters your data. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Wed Nov 12

How does rubber stamp pervert the data? Define this. Gary Gaugler gary@gaugler.com Wed Nov 12

The rubber stamp (clone tool) works by replacing the image pixels with other pixels taken from elsewhere in the image. If we consider each pixel of a micrograph (or other picture) taken for scientific purposes as a data point, replacing pixels with pixels from other pixels replaces data with (wrong) data. Presumably getting rid of a constant set of pixels (a blob on the camera, for example) from an image by subtracting a background image will let any pixel value not subtracted by this method show through. Any pixel of a value that is subtracted as part of this manipulation will not show; hence the requirement that it be reported as an image manipulation. This is still preferable to replacing "unwanted" pixels with pixels from somewhere else! Get ready for the requirement that the "original" of any image that is published be available for inspection, and that all conditions of the acquisition of that image be stored with that image. It's coming. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Wed Nov 12

I've not done it with EM data for standard photo manipulation I convert the ref image to negative and simply overlay - objects that appear in both images then magically vanish. Ian Portman i.j.portman@warwick.ac.uk Thu Nov 13

The dirty optics 'Dirt' on a camera chip is normally fairly in focus. I am assuming you can't simply remove the camera and clean the 'chip' [normally a cover-slip thickness piece of glass covering the actual chip on dedicated microscope cameras]. This is a delicate procedure and I use ether and pure cotton wool on a stick and clean in a circular motion so as not to drag dust in from the edge of the fitting. Canister Air jets can be used but I mostly use a large hand puffer after getting nasty propellant sludge all over this delicate glass when using an 'invertible' duster spray many years ago. If it's a camera that takes a lens [i.e. SLR or compact] take some pictures away from the microscope to check for dust. If the camera's a £12k Hamamatsu, get in an engineer if you're really worried about doing it yourself; his insurance can cover it, cost about £300 to £600, full microscope cleaning included. My advice is to rotate any optics that can rotate, like cameras, filters & objectives, and if the dust rotates with it then you have found the problem. Normally however 'dust shadows on an image' is because the condenser aperture diaphragm iris [the one that isn't the condenser field diaphragm iris that's adjusted for Koehler illumination] has been closed down which decreases resolution/brightness but increases contrast and depth of field. Increases in the latter naturally brings in all the dust on the microscope internal optics producing an image identical to the one you describe [dusty shadows all over the place]. A dark shadow in the centre could be badly adjusted Koehler illumination, and here the phase rings are the culprit. There are plenty more other possibilities, particularly with oil immersion, but the above is the most likely. Hopefully it's not due to damaged optics. The photograph. Removing dirt from the image via Photoshop is pointless really as you can't recover the information under the shadows unless you have another identical picture of the sample with the dust in different place. All you can do otherwise is copy and paste similar looking areas nearby over the top of the muck. I would do that for photos of the kids' faces and for web site images but not for scientific publication as the information you are adding is false. My favorite tools are the lasso and magic wand, with copy, drag and paste over, with a hint of smudge over any obvious borders after the layers are merged. But this is mostly art & aesthetics not science. As you say, you can 'background subtract' an image of an empty field from the specimen image you have just captured using the identical optics/light intensity settings. This markedly reduces the effect of uneven illumination [say if you are using a 1x objective with no condenser] but it will not remove dark black dust shadows if they have obscured the specimen detail. Naturally you have to do this live when you are taking the photos. It can't be done days later unless the empty field 'subtract' image was also saved. Our image acquisition software does this 'background subtract' automatically for us with varying degrees of success so I don't really have to think about how it does it on a pixel to pixel level. With gradual uneven lighting you can often artificially generate your own background subtract image, but this won't work for your 'dust' shadows. The Molecular Expressions website has some info on this: http://micro.magnet.fsu.edu/primer/digitalimaging/backgroundsubtractiontoolkit.html Keith J. Morris kjmorris@well.ox.ac.uk Thu Nov 13

Not all "dirt" is 100% opaque, so reference images of a blank field with illumination off for one and on for another can give you both a dark reference and a bright reference that can be used to subtract noise and normalize the response of each pixel. Digital-Micrograph (usual disclaimer; no commercial interest, just a user) provides for this in their software, and I assume that there is an equivalent function in whatever software you use. If the shadows

are not too strong, normalizing is acceptable--with appropriate documentation, as mentioned by others--but if the shadows are too dark, the signal/noise ratio may be inadequate in the affected pixels. In the limit of completely opaque dirt the normalization will multiply the pixel values by a very large number, or will replace the pixel value with a constant, depending on how the normalization program has been written. Another method, which may not work if you are taking images of a dynamic process, is to take two images, one of which has the specimen offset somewhat with respect to the other, and then you should have the correct intensity available in one of the two images, so you can cut and paste the appropriate areas. Again, save the originals and document the processing. Bill Tivol tivol@caltech.edu Thu Nov 13

I would like to draw your attention to a symposium being organized at M&M 2009 where the title and description are Raw Data and Metadata: Comprehensive and Ethical Collection, Storage, Manipulation and Retrieval of Images. We are in an era where it is becoming essential to record comprehensive meta-data along with our microscope images. Meta-data can be used for documenting experiments to make the data useful and meaningful to others if we are required to make raw data publicly available. We also need to be concerned with the ethics of post acquisition image manipulation - reliable, secure meta-data can help prevent misuse of images. Data mining is attracting attention with the advent of the "semantic web". We seek presentations and posters on all aspects of data collection, storage and retrieval especially in a core facility setting. I would be delighted if the session was oversubscribed with contributions, and just as delighted if the room assigned to the symposium was overflowing with attendees! I would like to strongly agree with the point that Tina made yesterday. Think of an image as a set of numbers - for a monochrome image this is simply an x coordinate, a y coordinate and an intensity value. Anything that changes those numbers should be carefully considered and always reported. But then of course we get into what was done to a signal before we, as users, even get to see it as an image, hence the symposium and thoughts about metadata and what really is the definition of "raw data" See you in Richmond for lively discussions. Christopher J Gilpin christopher.gilpin@utsouthwestern.edu Thu Nov 13

**TEM – image distortion**

*I've noticed for some time now that image in our H7500 is distorted. When I look at the grid bar it has an "S" shape. The center is straight but the ends are slightly bend in opposite directions. It is visible at low to mid range magnifications. I am looking for advice what is the cause of that problem and how to correct it. Dorota Wadowska wadowska@upei.ca Tue Oct 28*

Rick Lawrence at SDSC has investigated this in detail, and he has written a program, TxBR, to correct for it and other distortions. The one you describe is called spiral distortion, and it is more bothersome at low magnification and especially in very large fields of view. Bill Tivol tivol@caltech.edu Tue Oct 28

A very weak electron lens causes pincushion distortion, viewed on the low side of the diffraction point. Moving slightly to the high side of the diffraction point you will see barrel distortion. Manufacturers balance these two distortions to make the image within the old fashioned "photo area" truly square. The result of balancing one lens with pincushion with another lens with barrel distortion

is known as "S" distortion or "anisotropic" distortion. The image at the very edge of a large diameter viewing screen will display this distortion, its normal. Steve Chapman protrain@emcourses.com Tue Nov 11

**TEM – comparison with STEM**

*As I have no direct experience with either STEM or TEM, I would appreciate comments on the following: 1) Resolution is better with TEM but atomic resolution is still easily obtainable with STEM, as long it is not 1 angstrom resolution you are looking for (structure of carbon nanotube or graphite for instance is easily seen with STEM). 2) STEM is much more user friendly than TEM, especially for people who have experience in using SEM. 3) STEM does not require as extensive sample thinning as TEM. TEM needs a wafer always thinner than 100 nm, STEM wafers can be 1 micron (but not for atomic resolution, obviously). 4) STEM allows more analysis to be done because you don't have the lens stage below the sample, so it is easier to do EELS, electron diffraction, EDX, etc. Patrick Lemoine p.lemoine@ulster.ac.uk Wed Nov 5*

1) Since resolution depends on both the initial size of the incident beam and the extent to which it spreads as it passes through the specimen, a small beam, say 0.2 nm, and a thin specimen will allow high resolution STEM, and that resolution can be as good as TEM for a thickish specimen. 2) I agree that STEM would likely be more user friendly for those with SEM experience, which I do not have. I do not know, however, whether there are subtleties in STEM that would be missed if one treated STEM imaging in the same way one did SEM imaging--I also have no STEM experience. 3) Again, I'm not sure that just using thin enough STEM specimens will give good results; that depends on what you want and the nature of your specimen. For thick specimens, scattered electrons will generate signals from parts of the specimen that are outside of the area of the incident beam, which might not be relevant to your work, but which could easily have adverse effects on resolution. One may find that one needs to thin a STEM specimen to the same extent as a TEM specimen in order to get good results. 4) Absolutely, more and different kinds of signals are usually collected in STEM than are in TEM, although a TEM/STEM machine will be capable of getting all the info available to STEM. A dedicated STEM usually is capable of better STEM performance than a TEM/STEM. It is, however, pretty easy to get EELS, ED, and EDX info from a TEM, as well as bright-field and dark-field imaging. Bill Tivol tivol@caltech.edu Wed Nov 5

**SEM – backscattering detector image formation**

*I need a simple explanation of the way a backscattering detector produces compositional vs. topographical images. I know it is a subtractive process where, if you had a 2-section solid state detector you would have A+B for COMP and A-B for TOPO. But what is actually happening to A + subtract the elemental information leaving only the topographical information? Debby Sherman dsherman@purdue.edu Fri Oct 24*

The elemental information isn't really subtracted from the signal. Topographic information results from the line-of-sight nature of backscattered imaging. Since BSEs are not drawn to the detector as SEs are, only those areas of a specimen directly "visible" to the detector are sensed. If the entire detector surface is used to receive the incoming BSEs, the surface will look more-or-less "evenly illuminated". This is because if BSEs from region A of the sample reach segment 1 of the detector in greater number than they reach segment 2 of the detector, the resulting signal is still the same (or nearly same) strength as if the BSEs from region A reached both segments equally. This is because the output of the segments is read as if they were coming from one detector segment and not 2 (or 4 or ... ). The output signal is summed from the entire detector area. So, by dividing the detector surface into 2 or 4 (or ... ) segments, the signal from region A reaching segment 1 can be read out separately from the signal from region A reaching segment 2. Subtract the signal of segment 1 from the signal of segment 2, and the difference in signal due to greater or fewer BSEs "seeing" the detector can be determined and presented as a topographic image. A corollary of this is that the topographic image can be changed by dividing the detector into smaller segments (e.g. 4), allowing different pairings of segments. So, if there are segment 1=> 4, then (1+2) - (3+4) will give a different image than (1+3) - (2+4). The compositional information is still there, it's just lost in the topographic information. Set the detector to read out as a single segment, and the topographic information is now lost and the compositional information can be seen. With the caveat that if there is much topography, the compositional image can be compromised or lost in the signal fluctuations caused by the topography, with a detector set to "compositional" imaging. So, BSE samples for compositional imaging are generally polished. Although I do get good compositional imaging from unpolished, low-relief samples. This gives me a chance to plug one of my favorite SEM books: "Scanning Electron Microscopy A Student's Handbook" by Postek, et al., Published & sold by Ladd Research, $39. Yes, it's old and yes it really needs to be updated and I keep hammering at the Ladd people to get on Mike Postek about doing that and encourage everyone else to chime in. Goldstein, et al. is an excellent book, and where I go for the really in-depth stuff, but Postek is the best basic reference and a good teaching book. Philip Oshel oshel1pe@cmich.edu Fri Oct 24

Phil has given a pretty comprehensive explanation of backscattered imaging but one point worth making is the way the backscattered electron detector imaging relates to our eye's view. Everhart-Thornley detectors receive an image as if the detector itself was lighting the structure i.e. surfaces pointing towards the detector are brighter than those pointing away. Backscattered electron detectors of the scintillator type "light" the specimen from overhead with a "lighting" bias towards the photomultiplier due to an off balance detection. Solid state backscattered electron detectors also "light" the image as if from overhead, producing an image with some topography even if you take all of the signal sections. But one big advantage of the detector is that it produces an image similar to the specimen being viewed in a light microscope; also lit from overhead! Those who do not have a multi segment detector (are there any left) may, as in an Everhart-Thornley detector, obtain higher topographic contrast by simply tilting the specimen. It is interesting that in the more the recent past everyone has been asking about how we measure surface roughness, this too is better viewed in backscatter. Why do people ask this question, well to claim a human face is made smoother by their method. The things we do for science! One more point, whilst teaching in Australia one of the lecturers commented "We do science, not tradition!" what a

superb saying as it seems to me most people go for tradition? I have a similar saying "Microscopists are scientists too, so experiment!" It's nice to see Debby is clearly experimenting but how many others follow? Steve Chapman protrain@emcourses.com Fri Oct 24

I take the opportunity to ask a question more about two sectors BSE detector imaging. As far I understand the way it works, the topo (A-B or B-A) image is not a true rendering of the topography of the sample, but more the derivative of the topography. If one looks at a sample with flat steps, only the border of the steps will apear as a dark or bright contrast. The steps themselves will be all more or less the same gray, flattening the topographical aspect of the surface. One don't see the topography, but more it variations. For more than twenty years I have worked on SEM's, I cannot remember a sample/situation where this "topo" image was the best way to answer a question (in contrast with the compo/A+B mode, whose interest is very evident). So, I would be interested in some examples where this type of images brings something more than the Everhart-Thornley detector. Jacques Faerber jacques.faerber@ipcms.u-strasbg.fr Fri Oct 24

Picking up on Phil's explanation, BSE signals are the result of the intensity due to composition modified by the geometry of the location. For small tilts around horizontal, you might say that the overall intensity pattern of the BSE emission is the same, but it is shifted off center toward one portion or another of the detector. Thus the various segments would no longer give identical signals. The difference would be due to topography. So is a TOPO-mode signal void of composition information? I doubt it. I would expect the fluctuation in signal due to topography in copper to be much larger than the fluctuation due to the same topography in silicon. Proportionally, the changes in signal may be similar, but the absolute changes in signal would be less for Si than for Cu. If similar topographies were present for both phases in the same image, I would expect the Cu side to show more contrast than the Si side. The average brightness should be the same for both sides. So how is it that a COMP-mode signal still shows topography? You might say that the angles are no longer small and the BSE pattern has not simply shifted from one segment of the detector to another, but has actually moved off the detector so that overall intensity decreases. Also, the intensity of the entire BSE signal may be altered by the change in geometry. Now, the topographic information may not be very evident or easy to interpret in COMP mode. It is like a coaxial lighting arrangement. I can tell that my intensity is down, but I cannot tell which way it scattered. To do that, I need to invoke some measure or other of topographic mode. I may not need to go all the way to A-B, maybe just A would be enough. Now our older JEOL does not allow total freedom of selecting the quadrants, but it does allow us to combine signals. Thus, COMP+TOPO = (A+B)+(A-B) = 2A. Warren Straszheim wesaia@iastate.edu Fri Oct 24

Well, we are both on the same wave length here! In my 40+ years with SEM I too have not seen a "topographic" imaged, gained by subtracting signals, that solved a problem and I am working with people's problems almost all of the time. In many consultancy tasks, relating to failure analysis, we are very often using the BSE image in its so called compo mode to solve problems, as the best information about a failure is not always on the surface. We are also using BSE detection, as I said earlier, because it reduces the



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high contrast differences sometimes dominant in rough fractures; even at what I would call the correct kV! This also ties together the light microscopy with the SEM. Hope this helps? Steve Chapman protrain@emcourses.com Fri Oct 24

I too will be interested in the answers. The only advantages I know of for using topographic BSE imaging are: 1) It can be a good way around charging problems. The imaged BSEs have nearly the same energy as the beam electrons, so they will be less affected by specimen charging. In the Old Days before environmental and variable-vacuum SEMs and gas in the chamber to bleed off charge, BSE was the only hope for imaging chargey samples that could not be coated (like museum specimens). 2) TOPO imaging can be used to get a "sort of SE-like" image of a specimen on which BSE imaging or E/WDS is being done. Usually it's difficult to get SE images of such subjects, since the specimens are either uncoated or carbon-coated and so chargey, and the BSE topographic image can be collected without changing any of the operating parameters -- spot size, aperture, working distance, etc. and so on. Just flip the detector switches from COMPO to TOPO and maybe rebalance the contrast and brightness. Otherwise, I'm in your camp, SE imaging is better. Mind, it's really cool when you can do both at once, which can be done with the right BSE detector (like an Autrata). Philip Oshel oshel1pe@cmich.edu Fri Oct 24

I do not agree that SE images are better but I am sure you would modify that to say there is a technique for a problem? Most modern systems offer dual display so we can all enjoy comparing the Everhart-Thornley image with the BSE image without complicated image mixing boxes; progress? Steve Chapman protrain@emcourses.com Fri Oct 24

I'm not sure I follow you. I thought I was agreeing with you. I do think SE gives higher resolution, and resolution being the sine qua non of microscopy, they are "better". See the SEMs on: http://www.ansci.wisc.edu/facstaff/Faculty/pages/albrecht/albrecht_web/Programs/microscopy/gallery.html These were taken with an Autrata BSE, which produces near-SE quality images at 5 kV. Or even 3 kV. Good as they are, the SEs are still better. Keep in mind, the BSEs are also coming from a larger analyzed volume, and so have lower spatial resolution that do SEs. Even with the best BSE detector. There is indeed a technique for a problem. That's essentially my point with using BSEs for imaging chargey samples, or for TOPO imaging of samples primarily used for COMPO BSE or x-ray. As far as I know all SEMs offer dual display. X-from the JEOL 35 I learned on years ago to now. Only SEM I've ever used that didn't have dual display was a Cambridge Mk IIa. Philip Oshel oshel1pe@cmich.edu Fri Oct 24

Sorry for the misunderstanding! Yes, I too use 5 kV BSE with whatever the client has as a detector, but I think my real point is resolution or information? On many courses, the clients think that they want higher resolution but 7 times out of 10 they are really talking about information. I believe, in many of the cases that I investigate on behalf of clients, it is actually the combination of Everhart-Thornley data and BSE data that finally solves the problem. My personal belief is that you should look at any sample with as many views as possible, SE, BSE and kV variations all of which build towards the final understanding of the specimen. Even doing crazy things like "it still charges at 2 kV" and going up to 15 kV in BSE often brings success. With Field Emission the problem of resolution is fading away and searching for the best presentation of the critical information is becoming the main goal. Steve Chapman protrain@emcourses.com Fri Oct 24

**SEM – backscatter detector**

*I have a Robinson Backscatter Detector attached to a Hitachi S-3500N SEM and I'm having a little problem with the images it's giving me. I'm analyzing diesel soot filter cross-sections and the channels of the filter in the images are lighter on one side than on the other. Even the pores in the filter are "illuminated" on the right side and tops of the pores. I've tried aligning the aperture and that wasn't the problem. I've tried increasing the beam current, as the Hitachi technician suggested, but that didn't completely solve the problem. He seemed to suggest that this may be normal with the Robinson detector, but I'm having trouble with the analysis with the illuminated sides. I know that it isn't the actual sample that has some elemental distribution on the sides because when I turn the image it's always the right side that is illuminated. Has anyone else had this kind of problem, or does anyone know the cause and solution to this? Kristi Majni kristi.majni@basf.com Tue Oct 28*

Yes, your engineer is right. That is normal behavior for a Robinson detector. We have a Robinson on our Hitachi S-2460N. It is mounted on the left side of the chamber. The left side of the detector seems to have a bit more area. Therefore, the image appears as if illuminated from the left and the right sides of the holes appear a bit brighter. Similarly the left sides of bumps appear brighter. Those comments are for the case where the raster is rotated so that things on the left side of the chamber appear on the left side of the screen. If you were to rotate the raster, the bright and dark areas should rotate around. If you rotate the sample (while leaving the raster set), the shading should maintain its orientation and the right side of the holes should remain light. Even in cases where there is no topography, we still see some offset in the brightness of the signal towards the left. I don't know if that is due to the center of the detector being shifted or the detector being a bit thicker and closer to the sample on that side. For that reason, we purchased an after-market solid-state detector. It mounts coaxially with the beam and avoids shading. It is also more sensitive at low voltages. However, it is a bit slower. The Robinson is a good detector and useful at faster scanning rates, but we rarely use it. Warren Straszheim wesaia@iastate.edu Tue Oct 28

That's an interesting situation. I don't see any difference in brightness from side to side unless there is some topology effect via the specimen and where the beam hits. The scintillator collector relies on the plastic light pipe to transport photons to the PMT. In its geometry, the furthest side of the scintillator is further from the light pipe feed to the PMT. But the difference is rather small it would seem to be an issue. If the scintillator is unevenly dirty, that will of course affect brightness across the scan. The detector ought to always be mounted on the side that supports the highest amount of specimen tilt. If the specimen is tilted, what happens to the image? Also, what does the SE image look like relative to BSE? If a scan is made of Au on C, does it also have uneven brightness? Strange. Gary Gaugler gary@gaugler.com Tue Oct 28

The Robinson Backscatter Detector will show directional preference due to the geometry of the detector itself. Physically it

is shaped like a fork with one end open and cannot detect electrons in the open portion of the detector. Hence; the overall detection efficiency is skewed toward the base and features in that direction will be more intense. Unless you block an equal portion of detector shape and area on the base side it will continue to exhibit these tendencies. Fran Laabs fclaabs@iastate.edu Wed Oct 29

**SEM - Coating for focused ion beam (FIB) SEM**

*We recently have acquired a Zeiss Neon FIBSEM with FESEM capabilities. I was wondering if anyone could give me any advice on a suitable coating unit. We have to replace our old gold sputter coater so I was hoping to buy something that could do both high resolution (platinum?) coatings and regular gold coats for SEM. I have read about osmium plasma coating but that is beyond our means at the moment and I am a bit worried about the OHS issues. Is platinum the way to go? Does anyone have any good/bad experiences to point me in any particular direction before I commit to a purchase? Elaine Miller e.miller@curtin.edu.au Thu Nov 20*

I have 2 FESEMs and use iridium for everything, including my FIB work. I will sometimes use evaporative carbon for FIB work, especially if I need to image using the ion beam extensively (carbon's low sputter yield means it will stay on the surface longer). If I need to do thickness measurements of thin layers, I put down a layer of carbon before I put down my metal deposition to make the cut. The carbon layer makes it easier to separate the surface metal from the metal I put down. Becky Holdford r-holdford@ti.com Thu Nov 20

**SEM – active and passive acquisition**

*Can someone give me the idiot's guide to the differences between active and passive image acquisition solutions for SEM's. I think I know that passive systems use the scopes controls to steer the beam, active ones add their own scan generator. Maybe someone can flesh out that explanation to include some details (not too complicated) and the advantages and disadvantages of each solution. Can you guess that I have to explain this to a class? My typical arm waving strategy might not work. Jonathan Krupp jkrupp@deltacollege.edu Mon Dec 1*

Yes, you've got the basic gist of it. The passive system reads the X and Y positions, giving a location, and also reads the Z (video) giving a quantity for the location. Continuing on, it builds the image in memory point by point, line by line. These are the easiest systems to install because all you do is tap the X, Y and Video signals. This system will also give you any data that your microscope normally writes on the images, so things like mag, kV, WD, etc. are automatically saved in the image. The active system requires one or more relays to be installed that will connect the SEM (mag, CRTs, etc.) to either the scan generator in the SEM or the scan generator in the active digital imaging system. The active system outputs the X and Y positions to the SEM and reads Z, thereby building an image. Although you lose the data your SEM would normally write on the image, you have more freedom to determine your image size and collection speed. Most SEMs don't scan more than 2000 lines in record mode, but the best systems can take 2000 lines on negative film and do fairly impressive enlargements to 10"x14" or bigger. They can do this because the individual lines are fairly fuzzy and the horizontal portion is usually analog. Going to digital and, say 2kx2k, a 10"x14" is not going to look as good. However, most of the active systems will collect at least up to 4k × 4k (some considerably larger), which will match or exceed the best recording systems at 2000 lines. Of course, an active system also allows an EDS system to look at an area, then go back to specific spots and do further analysis. An active system is also the basis for most EBL (electron beam lithography) systems. A passive system is a very good (and less expensive) replacement for Polaroid film. An active system will let you go beyond film, but will require more input from the user. Ken Converse kenconverse@qualityimages.biz Mon Dec 1

Active capture is like attaching an EDS system to the SEM and having its scan controller move the beam over the desired area. The SE detector signal output is digitized by the capture system as does an EDS imaging system. Normally, the SEM's scan generator drives the beam but in an active system, the separate capture unit drives the beam. The setup procedure must be done one time to calibrate max X and max Y voltages to get the captured aspect ratio to match that of the SEM. The next procedure is to set contrast and brightness in the capture system to match what is seen on TV display. The capture system will allow different x and y pixel dimensions as well as dwell time per pixel. This is like different slow scan speeds on the SEM but not always with allowed filtering amounts. Passive uses the SEM scan generator to scan as normal. However, the capture system taps into the X signal, Y signal and usually the blanking signal. The active mode blanks the beam by itself to delete retrace lines. Both modes sync to power line to eliminate herringbone interference. Bit resolution is determined by SEM scan speed in passive mode but by



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the user selection in active mode. The bit depth is at least 10 to 12 bits in either mode and is determined by hardware. In either event, the system needs calibration. If the A/D converter and buffer do say 4K × 4K pixels, the record CRT is for 4×5. So the x and y limits need to be adjusted in either mode one time. Active is way more versatile than passive but also more expensive. Passive can also tap into main TV CRT if it has the NTSC RS-190 signals. Some makers do PAL as well as both modes. Gary Gaugler gary@gaugler.com Mon Dec 1

**EM - $SF_6$ detector**

*I am about to install a TEM that has SF6 in the HT tank. I have read that an SF6 detector should be used to continuously monitor the air in the room for leaks. Is this commonly done or is the likelihood of a leak so remote that it is unnecessary? There is a big difference in the price of a detector that monitors continuously vs. one that is used for occasional use - such as when the filament is replaced. Wed Nov 5*

A lot depends on the size of the room and the air circulation that you have and local regulations. $SF_6$ is more of an asphyxiant than a toxin, and since it is quite heavy, any leaks would accumulate from the floor and not be breathed until the room filled up to the height of your head. You need to find out the volume of the $SF_6$ in your tank and do a simple calculation to see how far it would rise in your room. We have two HV systems, one of which developed a slow leak after ~12 years (the only effect was that we could not turn on the HT until it was re-charged) and the other that has not leaked in 11 years. We have an oxygen sensor in one lab that our safety people insisted on and one for nitrogen from the EDX system, but it is ~5 feet off the ground and would not detect an $SF_6$ leak. The old system once required service, and the $SF_6$ was removed by venting into trash bags and carrying the trash bags outside. It only took a few bags to complete the task. John Mardinly a.mardinly@numonyx.com Wed Nov 5

There should be a manometer (or is it called a pressure gauge?) continuously displaying the pressure in the tank. I consider it enough to indicate a leak and take action if necessary. Just check the pressure each time you enter the room. A far as I know, a TEM room should be air conditioned at least to avoid temperature fluctuations. Just make sure that the air is pumped from the floor level and not from the ceiling and the risks should approach zero. I must also say that the volume of this chamber is pretty limited too. Stephane Nizets nizets2@yahoo.com Thu Nov 6

When the room for our microscopes was "designed" by the building architects, they decided that the floor-level $SF_6$ exhaust ports would make swell A/C return ducts as well. There were only two serious flaws with this. First, the A/C inlet dropped cold air through a special laminar flow duct, and the air continued to the floor, under the curtains, then out the $SF_6$ duct, never coming close to the column, which sat in warm dead air. Second, the quantity of the exhaust caused a high level of acoustic vibrations in the room. The Provost was very generous, spending >$100k to retrofit the room, which the engineers determined needed 60 $ft^2$ (~6 $m^2$) of return duct area to meet both the temperature stability and air flow velocity specifications. The $SF_6$ tanks for our TF30 and T20 are ~2 m tall by ~0.7 m diameter at a pressure of a few bar, which is the equivalent of ~3 $m^3$. The smaller room with $SF_6$ is ~36 $m^3$, so the total $SF_6$ volume is a significant fraction, but, as was pointed out, $SF_6$ is an asphyxiant, not a toxin, so it would take a very high concentration to lower the oxygen content to a dangerous level. As long as there is enough air flow to mix the $SF_6$, there should be no danger, and if any did exist, there would be no problem unless one was constrained to breathe near the floor, so it would be dangerous either to be working near the floor or to have lost consciousness when the $SF_6$ tank emptied. That being said, however, I am strongly in favor of making the workplace as safe as is humanly possible, so I am glad we have the $SF_6$ exhaust ports near the floor when needed. Bill Tivol tivol@caltech.edu Fri Nov 7

**EM - CTF function**

*Does anyone have--or know where to download--a program to calculate CTFs and envelope functions at various values of defocus into which I can input the appropriate instrument parameters? Bill Tivol tivol@caltech.edu Tue Dec 2*

I know that CTFexplorer is useful: http://www.maxsidorov.com/ctfexplorer/ (from Andrea Nans and Henk Colijn) I am a Software Engineer/Researcher for a company called Media Cybernetics, working on deconvolution software. Not sure if it is relevant but I have been researching transfer functions of 3D aberration corrected STEM in order to deconvolve images from this instrument. Our product has some routines for the initial estimate of a CTF and then refines it as part of a blind deconvolution process. Demo licenses are available. (from Brian Northan) This one looks like it has all that you want but I've never tried it. http://ncmi.bcm.tmc.edu/homes/wen/ctf (from Craig Johnson and Angel Paredes) I recommend Dr. Earl Kirkland's online java code. http://people.ccmr.cornell.edu/~kirkland/ (from Huolin Xin). Bill Tivol tivol@caltech.edu Tue Dec 2

**EM – pump speed vs. ultimate pressure**

An old friend recently sent me the following question: "Would three different SEMs, with the very same leak size in the transition range, and same chamber volumes, but each with different TMP pumping speeds, say 100, 300 & 500 l/s, respectively, have the same ultimate pressure?" And since it is a matter of rather wide interest, I thought you-all might be interested in the answer, which is as follows: The simple answer to your question is no! The 500 l/s pump would definitely achieve a lower ultimate pressure. Justification of this answer is a bit more complicated. If you will refer to my book on Vacuum Methods in Electron Microscopy, there are two equations that are particularly relevant to this question. The first is equation 2.16 on page 48, which shows that the ultimate pressure Pu is given by the ratio of the rate of gas influx q to the speed of evacuation Se (i.e. Pu = q/Se). The second is equation 2.12 on page 40, and the graph on page 41, that show that the speed of evacuation Se is strongly dependent on both the speed of the pump Sp and the conductance C of the line connecting the pump to the chamber; that is: [Se = (Sp x C)/(Sp + C)]. Thus, since speed of evacuation is usually strongly dependent on the speed of the pump, equation 2.16 suggests that, for a fixed leak rate q, and everything else being equal, the ultimate pressure would be lower for the system with the pump having the highest pumping speed Sp; namely, the. 500 l/s pump. However, it is unlikely that all other things would be equal, because the smaller pumps have smaller diameters, and therefore are usually fitted with tubing leading to the chamber that is smaller in diameter than the larger pumps. This usual practice of using smaller diameter tubes with the smaller pumps would lead to a decrease in conductance C for those systems, and combined with the lower pumping speeds Sp of their pumps would normally cause the systems involving the

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smaller pumps to have very significantly lower speeds of evacuation Se than the larger ones, further increasing the difference in ultimate pressure attainable. From an operational point of view it is also interesting to note that most SEMs can be brought into operation at some operating pressure Po that is considerably higher than the ultimate pressure attainable in their vacuum systems. Equation 2.19 indicates that this pumpdown time t is inversely proportional to the speed of evacuation. Therefore, if all other things are equal, this pumpdown time would also be much shorter for the larger pumps. This is a matter of considerable importance to operators of the instruments, because it determines how long they need to wait before turning on the high voltage after specimen exchange, etc. Wilbur C. Bigelow bigelow@umich.edu Wed Dec 3

Your experience seems to be different from mine. All too often, when a manufacturer decides that a higher pumping speed is needed, they just add a bigger pump. But they do not redesign the pumping line to match. For example, they are unlikely to make a bigger pumping port into the sample chamber. The pumping speed (where you need it) and the ultimate pressure are then controlled by the conductance. The result of this - as your equation would show - is that putting on the bigger pump hardly affects the pumping speed. Alwyn Eades jae5@lehigh.edu Wed Dec 3

**EM - plasma cleaner**

*We recently purchased a Plasma Cleaner (Southbay Technologies, model PC2000) and I am trying to find out what is the best O2/Ar ratio for the plasma composition. Domingo Ferrer domingo@mer.utexas.edu Sat Dec 6*

The chemistry of the plasma and how the potential cocktail of gases used in them cleans a sample varies dramatically between the various commercial units. Every unit that I have used works well using the manufacturers recommended conditions, so you should always start there. However, you do not have to use both O & Ar in a plasma cleaning system to remove hydrocarbons. In most cases in my lab I start using only pure Ar and only resort to adding O when the contamination is severe. The model of plasma cleaner you are using it is a low/medium power plasma and the cleaning process is slow (which is the mode I prefer). I would recommend starting at 10-15 W, for 10 minutes at about 200 mT of Ar. See how it works on your samples and then adjust the time/power/pressure/composition to optimize for your specific specimens. I have used a range of conditions for different materials and very importantly you should realize that you will likely need different conditions for different ways your specimen has been prepared. For example for nanoparticles on C films I never use O, as the plasma will attack the support film, however, a short low energy Ar plasma works well to remove any residual organics without completely removing the C film. On the other hand for electropolished samples with lots of organic solvents I frequently mix in Oxygen, or in very severe cases use pure O2. Remember start with your manufacturers recommendations and then tweek the conditions from there. Nestor Zaluzec zaluzec@microscopy.com Sun Dec 7

**SEM – oil shale rock sample preparation**

*I was wondering if any of the mineralogists on this list have developed a sample handling and SEM examination protocol for oil shale rock samples. I've only just received an inquery regarding the possibility, but I have never handled such a sample. Our SEM is capable of environmental chamber pressures (FEI Quanta), but the work will require BEI imaging and EDX spectra. I imagine the vapor pressure from these types of samples can vary from nil to extremes - how would one determine before possibly contaminating the column if any particular sample was going to cause problems? Michael Shaffer michael@shaffer.net Mon Dec 1*

I can't say I have a protocol per se, but I think you should be able to examine the shale without equipment worries. We purchased a VP-SEM years ago for use with concrete. We have used it with all manner of other materials including oily samples. I should probably point out that we do most of our work with BSE since an SE detector was not available for our Hitachi SEM. The situation might be different for the SE signal. We do probably 90% of our work in VP-mode since we routinely encounter insulating samples. We use 40-100 Pa of helium as our residual gas to bleed away charge. The helium scatters much less than air or nitrogen at the same pressure. We often sweep the pressure over a range to determine the minimum pressure required to eliminate charging. Since we are operating at a considerable pressure, we find that hydrocarbons are swept from the system. We have very little trouble with pump oil accumulating on the EDS window. By contrast, we need to clean the detector window on our other SEM (a conventional, high-vacuum scope) every 6 months or so as we see oil accumulating on the detector snout. My biggest concern would be with the vacuum "pulling" the oil to the surface of the sample. We see such an effect with embedded and polished samples where polishing oil finds its way between the sample and embedding medium. The vacuum pulls it to the surface and the oil runs over the neighboring material. I suppose that could happen with your samples. Maybe a higher pressure would minimize the problem. Maybe you could find areas less affected. Bottom line: I wouldn't hesitate to try it. Warren Straszheim wesaia@iastate.edu Mon Dec 1

**SEM - paper**

*I have a request for information on what if any applications the SEM may have with paper products such as currency, lottery tickets, suspected fraud and counterfeit documents. The basis for the inquiry is whether a law enforcement group might justify obtaining an SEM to assist in investigation of suspected tampered documents and lottery tickets. Debby Sherman dsherman@purdue.edu Fri Nov 14*

The first SEM ever sold was the KCA Smith SEM sold to the Canadian Wood Pulp and Paper Institute, back around 1956. If that is not an endorsement of SEM's for paper analysis, I don't know what is. John Mardinly a.mardinly@numonyx.com Fri Nov 14

A cardinal principle of comparative materials analysis applies here. One looks at the microstructure of a manufactured product to see what it is (for comparison with a designed or reference structure) and to see what the structure indicates about the process that formed the structure. In the case of the paper products mentioned, one should look at the type of fibers present, including special fibers with microscopic labels, the type and composition of the ink and other coatings, and the way the pattern is printed. I'm not very familiar with the analysis of banknotes, but I do have a lot of experience in analyzing (with AFM) the structure of holographic decorations, some of which might be used as security or authenticity labels. It turns out that there are a variety of creating these decorations and a knowledge of the different processes and their hallmarks could well be useful in a forensic or intellectual property investigation. Don Chernoff donc@asmicro.com Sat Nov 15