# NETNOTES

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**Selected postings from the Microscopy Listserver (http://microscopy.com) from 12/10/05 to 2/14/06. Postings may have been edited to conserve space or for clarity.**

### LM - Refractive index of organelles

*I am doing a research assignment for which I require the values of the refractive index of mitochondria, nucleus, ribosome, and cytoskeleton at various wavelengths. I have tried searching for these values, but have gotten unsatisfactory results. If anyone knows where I can get such data, please help. Ann <matthems@rose-hulman.edu> 06 Dec 2005*

Most biological macromolecules, whether lipid, sugar, nucleic acid or protein, have a specific refractive increment very near 0.0018, meaning that each 1.0 g of dry weight dissolved (or suspended) in 100 g of aqueous solution increases the averaged refractive index of that solution, or of that volume occupied by the molecular assembly, by 0.0018. Thus, if water RI is 1.3300, 1% protein (w/w) is 1.3318. Dry 100% protein would be about 1.58 (1.33 + .185), if the specific RI increment is 0.00185 (behavior is not reliably this linear though at high concentration). I learned about all this while doing interference microscopy of myofibrils about 25 year ago. In our lab we still have a usable Vickers M86 scanning microinterferometer microscope that the last user coupled nicely to a Macintosh program, but we haven't found a fundable use for it in the last 20 years. See Barer and Joseph (1954) Quarterly Journal of Microscopic Science 95:399-423, for a discussion of refractometry of living cells and info about the specific refractive increments of solutions of the different macromolecules, varying in reality from perhaps 0.0014 to 0.0020. For more references of possible interest, including parts 2 and 3 of Barer and Joseph (1954-55), see the reference list in Joseph (1981) J. Microscopy 131:163-172. I'm not sure anyone ever measured isolated organelles except for myofibrils. You can do it yourself using a phase contrast microscope and irrigating under the coverslip with an impermeant immersion medium of graded dilutions to give graded steps in RI, in order to see which RI matches out the contrast of the isolated organelle of interest, making them almost phase-invisible. The two media I used for matching A-bands in myofibrils of insect flight muscle and rabbit psoas were Percoll, and Limulus hemocyanin, washed and finally concentrated by ultracentrifugation in the buffer of choice, then incrementally diluted and subject to RI measurements in an Abbe refractometer (M. K. Reedy, C. Lucaveche (1984) Advances in Experimentaly Medicine and Biology 170:29-45). Percoll was more innocuous than hemocyanin on rabbit fibrils; insect fibrils were unchanged by either one. Barer wrote a more reader-friendly text on the method that might still give you what you want; I found it a pretty good read. The reference is R. Barer, in Physical Techniques in Biological Research. A. W. Pollister, Ed. (Academic Press, New York., 1966) pp. 1-56. Mike Reedy <mike.reedy@cellbio.duke.edu> 07 Dec 2005

### IMMUNOCYTOCHEMISTRY - Permount and FITC

*Anyone ever have problems using Permount and FITC? I was asked if there were any problems that could arise. Rod Nicholls <nicholls@post.queensu.ca> 22 Dec 2005*

One doesn't generally used organic solvent based mounting media with fluorochrome labeled specimens. The xylene in Permount would destroy the fluorescence. You need one of the many commercial preparations or Mowiol. Google for the recipe on line. Tom Phillips <phillipst@missouri.edu> 22 Dec 2005

### IMMUNOCYTOCHEMISTRY - bacterial permeabilization

*I am trying to permeabilize some Haemophilus influenzae fixed in 2% paraformaldehyde + 0.2% glutaraldehyde so I can immunostain an intracellular protein. My first attempts to permeabilize with Triton X-100 were surprising unsuccessful. I am interested in comments from anybody with expertise in permeabilization, especially in regard to the bacterial target. Tom Phillips <phillipst@missouri.edu> 13 Jan 2006*

It is probably not a really necessary question but what concentration did you try the Triton X-100 at? Did that have any effect on the ultrastructure? I believe that although Triton X-100 may dissolve lipids, in my experience at least as important is the effect Triton X-100 has on opening up mildly fixed cytoplasm, rinsing out some of the components that have not been really crosslinked. What I mean to say is: if the cytoplasm still looks hardly affected, it may well be that it is not the detergent's fault but maybe too 'strong' a fixation, so rather steric hindrance than membrane barrier issues. Jan Leunissen 13 Jan 2006

Very interesting question. The problem you are faced with is bacterial structure. *H. influenza* is a Gram negative microorganism. Gram negative cells have two phospholipid layers - the outer and cytoplasmic membranes. In addition, you have a layer of peptidoglycan between the outer and cytoplasmic membranes. To make it even more fun, there is frequently a lipopolysaccharide layer around the whole thing. You have to penetrate the LPS, permeabilize the outer membrane, traverse the periplasmic space (the volume between the outer and cytoplasmic membranes), cross the peptidoglycan layer and then penetrate the inner membrane, all with gentle treatment to allow the membranes to reorganize after removal of the detergent you use for permeabilization. Permeabilization will be difficult at best, and most likely impossible. Probably the only way to get something reliably into the cytoplasm would be by active transport. Having said that, knowing what I do about the structure of prokaryotic cells, I confess to having tried to permeabilize and do pre-embedding labeling on *E. coli* expressing a protein of interest (another gram negative bacterium). Didn't expect it, but tried anyway. I used Triton X-100 and saponin but neither one looked good. No luck either way. At the same time, I did LR White embedding on cells from the same broth culture. The fixation protocol included osmium fixation. I used metaperiodate followed by hydrogen peroxide etching and then did indirect immunoelectron microscopy with 12 nm gold for the label. I got beautiful preservation and highly significant labeling of the expressed protein in the *E. coli* and in the wild type bacterium from which the protein of interest had been cloned. In fact, the protein of interest was hypothesized to be membrane inserted on the basis of amino acid sequence and the labeling of the wild type cells was primarily associated with the cytoplasmic membrane. In short, I did minor modification to the standard LR White embedding protocol and it worked great. Paul Hazelton <paul_hazelton@umanitoba.ca> 13 Jan 2006

You might try microwave-assisted fixation. I have no experience with bacteria, but we used this successfully on nematodes, where the cuticle is otherwise a serious impediment. I have published a protocol using an older model oven, placing the samples at the "hot spot", while chilling the sample in an ice slurry to keep from cooking it. This also worked to fix worm eggs, getting fix past the eggshell. More recently we have been experimenting with a newer model oven, tuning down the power to only 70 watts, and using a cooling plate to keep the samples at about 20-35 °C. This is starting to work, but we have not published the protocol yet. We can irradiate continuously for many minutes without overheating the samples. We often pre-chill the cooling plate to force the starting temp down to say 10 °C, so that even after long irradiations (say 5 min bouts at 70 watts), we do not go too high in sample temperature. If you can find such an oven, it is worth a try. See: Paupard, M.-C., Miller, A., Grant, B., Hirsh, D. and Hall, D.H. (2001) Immuno-EM localization of GFP-tagged yolk proteins in *C. elegans* using microwave fixation. J. Histochem. Cytochem. **49**: 1-8. David Hall <hall@aecom.yu.edu> 13 Jan 2006

See: Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science. 2000 Mar 10;287(5459):1816-20. They did it with ethanol. When staining an antigen in an Anaplasma, I have also found that Triton X-100 does not work but methanol does. Michael Herron <herro001@umn.edu> 13 Jan 2006

### TEM SAMPLE PREPARATION – Embedding tissue culture cells

*We have embedded tissue culture cells grown on tissue culture glass chambered slides using Polybed 812, polymerized at 60°C for 2 days. We tried to separate the glass by using liquid nitrogen followed by a warm water bath, but the glass did not separate from the polymerized resin. Can anyone suggest an alternative procedure to remove the glass from the block? 17 Jan 2006*

I take a thin razor blade and carefully insert it around the edge of the slide between the chambers and the slide. This usually creates enough of a gap so the liquid nitrogen can get in and pop the slide off. The new chamber slides with thin cover slips are a little trickier. You need to remove the

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"sealant" with razor blades and then expose the glass side to liquid nitrogen holding the chambers above the surface. Virginia Crocker <crockerv@ninds.nih.gov> 20 Jan 2006

Next time, you should only polymerize for about 7-8 hours before popping the cells off. I then usually re-embed the pieces that come off and polymerize using standard times. Tom Phillips <phillipst@missouri.edu> 20 Jan 2006

You might try growing your cell culture on Thermaox coverslips, 13 mm size fits right nicely. After you have polymerized your block with the coverslip, they can be removed by simply setting the coverslip on a hotplate set to its lowest heat setting for about 30 seconds to 1 minute, the coverslips peels off easily and the cells remain on the resin block. I have not used this method on glass coverslips so I can not say what might happen to the thin glass. If you are using glass slides a quick couple of passes through an open flame and the block snaps right off. This does not damage the cell. Ron Austin <rausti@lsuhsc.edu> 20 Jan 2006

We grow the cells on a coverslip and remove them by hydrofluoric acid afterwards. Round coverslips are better and they will fit into the chamber. It works very well, although the HF is not very pleasant to work with. Michael Jarnik <m_jarnik@fccc.edu> 20 Jan 2006

We too use concentrated hydrofluoric acid to dissolve glass coverslips. If you use plastic Tripour beakers, plastic forceps, and plenty of rinse water, it doesn't cause problems. Since slides are thicker than coverslips, they may need may need longer time in the acid. Coverslips usually dissolve in 20 minutes, if all the edges are free of resin. To help the acid get at the free edges, file down the edges of the embedded slides using a metal file. Then immerse in acid under the fume hood in a plastic beaker. Check periodically by dipping in water and looking at the surface. Wear nitrile gloves and work under the hood. The surface should be smooth and shiny with no patches of undissolved glass which looks like an iceberg melting. Rinse well under running water and dry in the embedding oven. JoAnn Buchanan <redhair@stanford.edu> 20 Jan 2006

**TEM SAMPLE PREPARATION – making PVA and PVAH adhesives**

*In the past I have used PVA dissolved in ethanol as an adhesive. (i.e., spread the liquid on a surface, and let dry which would leave a very thin film that would become tacky when heated). That was then ... and now I'm not sure what "PVA" is. I thought it was polyvinyl alcohol, but it may be polyvinyl acetate. If anyone knows, please remind me. Michael Shaffer <michael@shaffer.net> 07 Dec 2005*

PVA stands for polyvinylacetate. PVAH is usually used to distinguish polyvinylalcohol although people tend to be lax on the terminology. The acetate may be hydrolyzed to produce the alcohol. Commercial products often contain partially hydrolyzed versions in order to tailor their properties to a particular use, so both species may be present. If you look at the various PVA and PVAH spectra in an infrared database, you will often find them arranged both by molecular weight and by degree of hydrolysis, that is, by the proportion of esterified to hydroxylated units. As you would expect, the intensities of the hydroxyl and ester carbonyl peaks vary tremendously from 100% PVA to 100% PVAH. The greater the PVAH content, the less the polar organic solvents will dissolve it and the more readily it will dissolve in hydrogen-bonding solvents like alcohols. The common form of "white glue" is an aqueous dispersion of PVA colloids rather than a true solution and when dried cannot be readily redispersed. Unless the molecular weight is small, most PVAs are not soluble in large amounts in any single solvent. Methyl ethyl ketone tends to work best. If you have used ethanol in the past it sounds like the material that you were working with may have been largely PVAH. John Twilley <jtwilley@sprynet.com> 07 Dec 2005

**MICROTOMY – Cleaning copper grids**

*We have inherited lots of copper TEM grids that are decades old. Many of them have a dark patina on them, and, on those grids, we have been losing our sections during alcohol-based uranyl acetate staining. However, the sections remain when we use water-based uranyl acetate. We have tried sonicating the grids in ethanol, acetone, or chloroform, none of which has solved our problem. Does anyone have any suggestions for cleaning them so that we will not lose our sections during staining, or should we pitch them? We really do prefer to stain with alcohol- based uranyl acetate, since it provides a more intense staining. Dotty Sorenson <dsoren@umich.edu> 03 Feb 2006*

I would try putting your grids into a drying oven for at least 5 minutes after you cut your ultrathin sections. After they are totally dried down, you can take them out. If you forget and leave your grids in the oven overnight, it doesn't seem to hurt anything at all. The heat seems to bond the sections to the grids very well, such that I've never had an experience of sections coming off the grids, regardless of the state of the grids. To clean my grids, I just dip them into concentrated sodium hydroxide for a few seconds, and then rinse them by dipping them a few times in distilled water just before I pick up the sections. It sort of etches the grids. Garry Burgess <gburgess@exchange.hsc.mb.ca> 03 Feb 2006

We clean our grids in acid alcohol, a mixture of 3% HCL in 95% ethanol. Cover the grids in a small shell vial with the acid alcohol, swirl intermittently for 30 to 40 seconds or so and rinse well with 95% ethanol. If the grids have a very dark patina, then clean for a longer time. You'll know when they are done because they'll have a shiny, bright copper finish. We usually clean many grids at a time, but you could clean them one by one by dipping the grid in the acid alcohol and then rinsing in a stream of 95% ethanol just before you use them. This works better on grids that have been cleaned, but have set around for a while, long enough to have developed a small oxidized layer. Dark patinas like the grids you have will probably need to be put into a vial and cleaned as described above. Greg Strout <gstrout@ou.edu> 03 Feb 2006

Try soaking a few grids in store grade ammonia for about 1/2 hr to 1 hr to see if the patina is removed at all. Rinse well in distilled water and see what happens. Scott D. Walck <walck@southbaytech.com> 04 Feb 2006

I've always cleaned grids by quickly passing through the flame of an alcohol burner. Quick and easy. Works on my old grids, though they aren't decades old! Diana van Driel <dianavd@eye.usyd.edu.au> 06 Feb 2006

We were in the same situation some years ago. We cleaned the grids in the following way: - put the grids into a small beaker (25ml) filled with 5% - 10% hydrochloric acid - let the grids to soak for some minutes, gently shake several times (at the end of cleaning the grids should be shiny gold) - remove the cleaning hydrochloric acid solution and wash the grids several times with distilled water - remove the distilled water as much as possible from the beaker and replace it with acetone and let stand for some time - pour the acetone with the grids into clean glass Petri dish filled with filter paper - remove the filter paper with the grids and put it into another glass Petri dish and let dry out the rest of acetone - that's all The troubles with losing the sections during the staining could be overcome by making the grids sticky: - put 10 ml of chloroform into 25 ml glass beaker - cut about 5 cm of 3M Magic Scotch tape and wash it in the chloroform - remove the rest of the tape from the beaker - put the grids onto filter paper and drop the "sticky solution" on them using Pasteur pipette - let the grids dry and use them for collecting the sections. Oldrich Benada <benada@biomed.cas.cz> 06 Feb 2006

**MICROTOMY – glue for serial sectioning**

*I was wondering if any of you could tell me what kind of glue is used to make Spurr's ribbon's stick together. I need to do serial sections and collect them all. Rhonda Allen <rra@stowers-institute.org> 03 Jan 2006*

I seem to recall that diluted rubber cement can be used. Greg Erdos <gwe@ufl.edu> 04 Jan 2006

For glue to get section ribbons to stick together, I like to use Weldwood Contact Cement (avialable at your local hardware store) diluted 1:1 in xylene. I picked up this tip somewhere a few years ago, but don't remember where. I assume you have trimmed the block to be a 4-sided pyramid with a flat top being the block face from which sections will be cut. To apply glue, use a small end of a toothpick, dip in diluted glue, and dab onto one of the 4 sides of the block face, either top or bottom relative to orientation of block during sectioning. I usually tilt the block face so that the side I choose is nearly horizontal so the glue doesn't run down the block side, or over the actual face of the block. Be careful so that glue does not get onto the block face itself. Work quickly as the glue will dry fast. Then reorient the block for sectioning. There is also a product out there called Tackiwax, available from EM supply companies, which you dab onto the side of the block face. You may or may not need to apply gentle heat near it to get it to spread evenly over the side of the block face. Gib Ahlstrand <ahlst007@umn.edu> 07 Jan 2006

In our lab, there was a person using a thin coat of dental wax successfully on the bottom side of a block. Melt the wax in a beaker and apply a tiny bit to a shaped block with a warm spatula. You may have to trim out excess wax. I have Tackiwax also. It came in a tiny bottle. It seems to me that it is repackaged dental wax without color. On the other hand, I may be wrong about this material. Ann Fook Yang <yanga@agr.gc.ca> 09 Jan 2006

Does anyone know if the various glues suggested vaporize in the TEM? If so, does this cause any problems? Dave Patton <david.patton@uwe.ac.uk> 09 Jan 2006

I am not sure about vaporization issues, but I have used another glue. Using a sharpened toothpick dipped in acetone, I have dissolved glue from 3M adhesive tape ("Magic Tape") to apply thin amounts of glue to the top surface of the block when trying to obtain serial sections. I have not noted any contamination problems, but clearly you want to refrain from getting any of this solvent on the cutting face. However, when collecting serial sections, glue should only be your last resort. When properly trimmed with a fresh razor blade (I like the chromium steel blades from "Wilkinson Sword Blades"), so that both top edge and bottom edge are parallel to each other and to the diamond knife edge, most Epon-like resins allow serial sections to stick together quite well. I don't know how well this applies to Spurr's in particular, but I expect the same results. The cleaner those edges are, and the more parallel they are to each other and to the diamond, the better the result. Acrylic resins like LR White or LR Gold tend to be much more brittle, and those would be more difficult to handle in series. For a more exhaustive discussion of serial sectioning, see Hall (1995) Methods in Cell Biology, *C. elegans*: Modern Biological Analysis of an Organism. Epstein and Shakes (eds) Academic Press. pp. 395-436. Dave Hall <hall@aecom.yu.edu> 10 Jan 2006

**MICROTOMY – making Formvar films**

*When using Formvar in ethylene dichloride, is there any way to make the films slightly thicker. I know when using Formvar in chloroform, you can just drain slower or faster to make films thinner or thicker. Is there something like this for the ethylene dichloride formula? I have tried several different times but so far haven't hit on anything totally satisfactory. <wong@msg.ucsf.edu> 21 Jan 2006*

We used these films for 30 yrs and controlled the thickness mostly by the percentage of formvar to ethylene dichloride. What percentage are you using? Judy Murphy <murphyjudy@comcast.net> 23 Jan 2006

Using a more concentrated Formvar solution should do it. If you only have the pre-dissolved Formvar, you could try either letting some of the solvent evaporate or double dipping. For finer variations you might try letting the Formvar drain at a shallower angle, which will drain it slightly slower. Bill Tivol <tivol@caltech.edu> 23 Jan 2006

**TEM SAMPLE PREPARATION – Negative staining pili**

*We have a few users studying bacterial pili using negative staining with PTA (pH 6.5). We have gotten some pretty nice images. But often times, we could not find pili even on the positive control. When we did see pili, they were not on all bacteria in the same sample. Is this a common phenomenon? Do bacteria lose their pili easily when the external condition is not favorable? If that is the case, what should be done to minimize the loss. Thank you in advance. Hong Yi <hyi@emory.edu> 09 Feb 2006*

We often have this same problem. At least in our lab, flagella and pili seem to hide when we're trying to find them and show up when we don't care if we see them or not. As a rule, they seem to be easily detached by rough handling, including centrifuging, and maybe by the staining process itself, since we often find them isolated from the bacteria on the grid. One group of researchers gets around this problem by growing the bacteria on carbon coated grids and staining them *in situ*. Check the Journal of Bacteriology Feb, 2005, p. 1173-1181 for images. For preparation details, this article references Brown *et al,* 2001. Immunocytochemical localization of HrpA and HrpZ supports a role for the Hrp pilus in the transfer of effector proteins from Pseudomonas syringae pv. tomato across the host plant cell wall. Mol. Plant-Microbe Interact. 14:394-404. I have not yet read the last article, so I can't comment on it. Randy Tindall <tindallr@missouri.edu> 09 Feb 2006

Rough handling may contribute to loss of pili, however the PTA itself is probably the culprit. Try changing the pH either up or down. Way back when, I did a study with rotavirus and various negative stains. Bottom line is that the length of time of staining, pH and concentration all contributed to the destruction (preservation) of the viral particles. If I remember correctly,

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the length of time was the most important factor. Also, try using either uranyl acetate or ammonium molybdate as the negative stain. Edward P. Calomeni <edward.calomeni@osumc.edu> 09 Feb 2006

I have been using 2% uranyl acetate to visualize bacterial pili. I have had to play with the time of staining and rinsing in order to get a good contrast to visualize the pili on different bacterial genera. I have noticed that not all bacteria show the same distribution of pili. I have also noticed that the way you grow the bacteria also affects visualization of pili. For our bacteria, I have noticed that I get far better results when I grow them up in stationary cultures without shaking them. What I have been seeing is that the bacteria behave differently when in aggregation versus solitude. This is more so owing to the several different kinds of pili each bacterial strain is capable of forming. If the bacteria you are working with produce different types of pili, then you will see differences between the bacterial cells visualized on the same grid. I have had the same strain of bacteria producing really small pili that were visualized only at a very high magnification. Vinod <nairvinods@gmail.com> 09 Feb 2006

**SEM SAMPLE PREPARATION – Critical point drying**

*What type of CO2 do you use for critical point drying? The $CO_2$ we currently have hooked up to our Denton DCP-1 is supercritical fluid extraction grade but it costs more than $750 (Canadian). I looked up some online sites where people mentioned that they use just standard $CO_2$ (in the US). I'm not sure what standard $CO_2$ is and what the equivalent would be here. Lizette Tuason 03 Feb 2006*

You don't need the really pure stuff. We use food-grade, and in one trial found it cleaner (less water, oil, and particulates) than the expensive, "pure" siphon $CO_2$. Just be sure to use a siphon-$CO_2$ (comes in a cylinder with a siphon tube, so you get liquid, not gas, coming out), and spend the money for filters and dehydrating sieves. Phil Oshel <oshel1pe@cmich.edu> 03 Feb 2006

**SEM SAMPLE PREPARATION - Magnetic etching**

*I am looking for information about magnetic etching and I found in this web site a quite old message about it. The thing is that I do not have access to the Metals Handbook that is recommended there. I would like to use Ferrofluids EMG 708 and 308 for visualizing the martensite phase transformed in a 304LN at the tip of a fatigue crack. My questions are: -The sample is electropolished before the fatigue test; do I have to chemically etch the surface around the crack before applying the Ferrofluids? How big should the applied magnetic field be to magnetize the martensite before applying the Ferrofluids? I have read in a paper that I have to dilute the Ferrofluids in water and to add a wetting agent. What is this wetting agent? What is its function? Sonia Mato Diaz <sonia.mato@upc.edu> 12 Dec 2005*

Sonia Mato asked about the use of Ferrofluid to visualize magnetic phases. She asked 3 questions. My responses are inserted after each one. Do I have to chemically etch the surface around the crack before applying the Ferrofluids? I think this depends on what resolution you want to achieve when you image the surface. I assume that the martensite phase is produced by the fatigue process, not the chemical etch. Therefore, I suggest try first without etching. How big should the applied magnetic field be to magnetize the martensite before applying the Ferrofluids? Try a strong disc-shaped permanent magnet, like the rare earth magnets sold by Edmund Scientific. Try to hold it about 1-2 mm above the sample surface. I have read in a paper that I have to dilute the Ferrofluids in water and to add a wetting agent. What is this wetting agent? What is its function? In our lab we use "Joy" brand liquid dish detergent as the wetting agent and dilute at least 10x. The purpose is to keep the Ferrofluid particles from clumping together. The particle size might be 50 nm or smaller, which allows high resolution imaging of magnetic domains by optical microscope and SEM. Magnetic domains can be imaged with or without Ferrofluid particles using AFM in Magnetic Force Microscopy mode. Don Chernoff <donc@asmicro.com> 13 Dec 2005

**SEM – image distortion**

*In our old SEM we found image distortion. Please see the image of Cu grid at http://www.kaker.com/300x.jpg. Can anyone help me to correct this error in our SEM. Henrik Kaker <Henrik.Kaker@guest.arnes.si> 08 Dec 2005*

We hope that someone with JEOL 35 schematics can answer your question specifically. Is the stage position moving during acquisition? If not, I would check the horizontal axis scan generator and coil drivers. From what

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I see, the waveform slope (magnification) is steady, but the zero point is drifting. It could be "zero point drift" either the scan sawtooth generator, the power drivers for the coils, or drift in an electrical x-y position adjustment. Since I don't have schematics, it is difficult for me to recommend specifics. Woody White <NWWhite@bwxt.com> 08 Dec 2005.

Check that the specimen is grounded. The stage grounding wire may have come loose or become broken. Confirm by directly grounding the specimen holder inside the chamber. The other cause could be loss of AC line sync. Gary Gaugler <gary@gaugler.com> 08 Dec 2005

The image shows a problem with orthagonality. I'm assuming that the specimen is not tilted. If that is the case, then an oscilloscope would show that the X signal has a significant amount of Y summed to it. I checked my schematics for a 35GF and there doesn't appear to be any correction adjustment for orthagonality. It would be located in the magnification section if present. Do you have a scan rotation and tilt correction module? I don't seem to have a schematic for it but this module could malfunction in such a way as to give you exactly what you are seeing. It could be as simple as a dirty switch or a stuck relay. Try turning the rotation function on and off, also try rotating the dial and see if the distortion changes. If it does, the problem is in the scan rotation, tilt correction module. If the distortion doesn't change, my best guess is that there is some cross-talk between the X and Y signals in the vicinity of the magnification section. It could be a cabling error, given the magnitude of the distortion. Ken Converse <kenconverse@qualityimages.biz> 12 Dec 2005

**TEM - LR White contrast**

*Does anyone have experience in contrasting LR White embedded thin sections with uranyl acetate and lead citrate? I am new to using LR White and tried halving the "normal" incubation times in uranyl acetate and lead citrate, but the sections are unusable - much too dark and full of stain deposits. Can anybody with experience with LR White suggest a staining protocol? Gerd Leitinger <gerd.leitinger@meduni-graz> 02 Dec 2005*

I contrast LR White thin sections with 3% aqueous uranyl acetate for 5 minutes followed by 3 minutes in Pb citrate (Venable & Coggeshall). Wash thoroughly with water after the uranyl acetate and with 0.01 N NaOH followed by water after the lead. I have found this to give good contrast, and I can still see the gold label easily. Leona Cohen-Gould <lcgould@med.cornell.edu> 02 Dec 2005

I have been using LR White embedded animal tissue for my research. After having immuno labelled the grids, I stain them with 5% uranyl acetate for 10 minutes followed by a 1 minute rinse in distilled water. I use a few pellets of NaOH in a covered Petri dish containing lead citrate drops and incubate the grids in lead citrate for 10 minutes. Thereafter, a quick rinse for 1 min in distilled water and view the grids after they have been air dried for about 5 minutes. Vinod Nair <nairvinods@gmail.com> 02 Dec 2005

You might be interested in several papers by S.-H. Brorson in the journal Micron, most from the 1990s, all electronically retrievable as PDFs. I recently became interested in these because of some unusual insights that Brorson develops about how raising the content of accelerator 2-8% or of propylene oxide 0-10% can reduce crosslink density of the final cured epoxy, and the relationship of this to rubbery-versus-brittle macro properties, polymer bonding to side-chains of embedded biomolecules, ultramicrotomy cutting quality, post-embedding antigen exposure, and comparison of immunolabeling with LR White. He reports that reduced cross-linking enhanced "antigen retrieval" (= antigen exposure to post-embedding immunolabels by incubation of sections in hot citrate) in thin epoxy sections, such that immuno-labeling for larger antigens could become as efficient as in LR White (although he notes less or little success with smaller antigens). Micron 29:89-95 (1998) is one of these. However, Micron 35:619-621 (2004) seems to hint that he may have given up the high-accelerator (8%) approach in favor of etching with citrate at 95-145°C and immunostaining at 60°C. I can't tell for sure, so I will copy this message to Brorson himself, hoping for his answer. You might also be interested in the obscurely published finding that quite good structure and antigen survival can be achieved after using uranyl acetate as a primary fixative: Fassel & Greaser, 1997, Microscopy Research and Technique 37:600-601. Mike Reedy <mike.reedy@cellbio.duke.edu> 06 Dec 2005

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**TEM - Colloidal Silica Particle Size Distribution**

*I was looking for help with a problem we have related to particle size distributions (PSD) with colloidal silicas as measured by TEM. We normally dilute a colloidal silica solution in an aqueous solution containing a few drops of nonionic surfactant to help disperse the particles (5-100 nm). After placing a drop on a copper grid (Formvar) we dry it at 100 oC for 10 minutes and place in the TEM for PSD. We are trying to automate the process with counting software but the software has problems identifying individual particles when they are touching one another. Does anybody have suggestions for a better solvent system or surfactant that might separate the particles better? Or is the drying process forcing the particles together and would a vacuum drying or other method work better? Mike Kamrath <mkamrath@nalco.com> 11 Jan 2006*

May I ask what you have done to ensure that your support films are hydrophilic? Carbon coated polyvinylformal supports become increasingly hydrophobic with age. We get good results by treating with spectro-grade acetone for 30 min prior to use. We have also used a short plasma-ash in air to improve wettability. Such treatments improve dispersion and reduces drying artifacts. John R. Minter <jrminter@rochester.rr.com> 11 Jan 2006

**TEM - Beam problem**

*I was having a brightness issue last week that I thought might be resolved with cleaning the Wehnelt assembly. Now when I energize the filament I get a very small circle on the view screen. It does not appear green - it looks like a dim reddish color. It also seems to respond to turning the filament knob but the condenser and deflection knobs do not cause any change. I have removed all apertures to see if there was a beam anywhere but no luck. <aetmicro@optonline.net>.*

What you see is the light from filament, not an e-beam. Whenever an electron gun is disturbed, in your case for Wehnelt cleaning, the gun tilt must be re-aligned, and perhaps the gun shift too. Both these alignments are mechanical on the Philips EM300. The users manual will help if the TEM is properly functioning. Green light or not, you must be able to tell whether high tension (HT) is present by watching emission current, and emission meter behavior: a) when HT turned on/off; b) emission setting changed while HT is on; c) and HV setting changed while HT is on. If HT is present, if filament glows, and if emission current behaves normally, do the following: a) select lowest magnification - I believe it is SC (scan) position; b) remove all apertures from the beam path; c) if no beam yet, turn lens currents off one by one and you must see the beam. Be careful not to burn the screen (keep filament under-saturated). Just turning off C1 and C2 will likely bring the beam back, and then work from that point on. Vitaly Feingold <vitalylazar@att.net> 13 Jan 2006

What you are seeing is the projected image of the hot filament (light). This means that although you have a hole down the column the electron gun is out of alignment. May I suggest the following: At no more than 60kV, switch off all the lenses and look for a very bright electron beam by adjusting the gun alignment controls. Once you find the beam, switch each lens on one at a time, stepping back if the beam disappears. Steve Chapman <protrain@emcourses.com> 13 Jan 2006

**TEM - Starting ion pumps without cooling water**

*Over the Christmas break we routinely shut our microscopes down to run only on ion pumps as our cooling water can be a little "temperamental". This year we had a power glitch which turned the microscopes completely off. To top it off our cooling water is out of action for maintenance until next week!! I was hoping to get all the ion pumps running on the microscopes to keep the vacuums at least a bit clean and I have managed to get this done on all but our JEOL 2010. Does anyone know if it is possible to get just the ion pumps running without needing cooling water on a 2010? Colin Veitch 03 Jan 2006*

I don't know the exact details of the arrangement of the vacuum system on a JEOL 2010, but in general there should be no requirement for having cooling water running while starting up or running a sputter ion pump. In fact, one of the principle advantages of these pumps is the fact that they do not need cooling of any sort for normal operation. What you would need to do is to set the valves in the vacuum system so that the backing pump evacuates the region of the vacuum system served by the ion pumps, then evacuate this region to a vacuum in the low $10^{-3}$ torr range. Then, follow the start-up procedure recommended for your ion pumps. You want to minimize the time the backing pumps are operated in the low end of this pressure range to reduce the possibility of contaminating the system by back streaming of oil

from them, otherwise, there should be no problem starting up the ion pumps. As soon as they do start you want to close whatever valves are available to isolate the part of the system they serve from the part evacuated by the other pumps. Once the supply of cooling water is restored, use the diffusion pump to evacuate the rest of the system to an operating level, and then open the valves to the ion-pumped region and resume normal operation. If you can get hold of a copy of my book on Vacuum Methods in Electron Microscopy (ISBN 1 85578 053 4) you can find the matter of backstreaming from rotary vane pumps discussed in Section 4.1.5 on p. 144. The general operating characteristics of sputter ion pumps is discussed in Sections 7.1.6, p 290 and 7.5, p. 322. I believe the operating procedure for the vacuum system described in Section 9.3 on p. 392 is very similar to that for the 2010. Good luck, Wil Bigelow <bigelow@engin.umich.edu> 16 Jan 2006

I too am not aware of any water cooling for ion pumps. When first pumping, they do get warm. But their controller will shut them off for a cool down. The main problem I have had in the past is getting them to start pumping. Initially, no pump current. This is fixed by heating the pump with a hair dryer. Then they fire and that is that. This is typical of the little 1-5L/m gun chamber pumps for LaB6. The larger 30L/m pumps don't seem to have this problem. Gary Gaugler <gary@gaugler.com> 16 Jan 2006

Hitting ion pumps with a hammer also gets them started. The hammer is also very good at knocking loose whiskers that form in the ion pump. John Mardinly <john.mardinly@intel.com> 17 Jan 2006

I forgot to mention that trick too. I usually use the handle end of a screwdriver. This also works for cold cathode sensors. Gary Gaugler <gary@gaugler.com> 17 Jan 2006

I was interested in the comments about banging on the sides of a sputter ion pump with a hammer or screw driver to overcome the startup problem that arises when a whisker or flake of titanium has formed and shorted out the path between the cathode and anode of the pump. Another way to treat this problem is discussed on p. 295 of my book 'Vacuum Methods in Electron Microscopy'. This involves allowing the pressure in the pump to rise slightly above 1 Pa ($10^{-2}$ Torr) and then turning on the high voltage power for a few seconds for three or four times. Be careful not to do this long enough to damage the power supply. Wilbur C. Bigelow <bigelow@engin.umich.edu> 21 Jan 2006

Yes, that might also work. However, if I just got through with an 8 hour bakeout, why would I want to degrade vacuum in the gun chamber? I would either never get the chamber pumped down or it would take a very long time to reach terminal vacuum. The idea is to get a vacuum as good as possible and then isolate the gun chamber before turning on the pump. Even so, startup current would be high. I've used the hair dryer most of the time. Then, sometimes whack the pump with the screwdriver handle. This is only for the small pumps. For some reason, they don't start as readily as the larger ones. Gary Gaugler <gary@gaugler.com> 21 Jan 2006

**EDS - Ge EDS detector**

*I am a bit confused on whether the Ge EDS detector can have the warming up cycle or not. Our detector is dead after one warming up. What is the mechanism of the failure if it warms up? I was never told that the detector should never be warmed up by the manufacturer technical support. Is it a common knowledge that Ge detector should never be warmed up once it is cold? Yan Xin <xin@magnet.fsu.edu> 16 Dec 2005*

Is it dead, or is it just the vacuum? I had a PGT Si(Li) detector that PGT told me could safely be warmed up for the Christmas holidays, so, after switching off the HV, I did so. However, on re-cooling a couple of weeks later, the vacuum had drastically deteriorated to the extent that the detector had to be re-pumped. It may be that some detectors, or some manufacturer's detectors, are more likely to be damaged by warming, regardless of the manufacturer's claims or advice. Ritchie Sims <rtch r.sims@auckland.ac.nz> 16 Dec 2005

It will be interesting to hear what the manufacturers have to say officially about this. Meanwhile, I can share our story. We had a Ge detector on an Oxford EDS system. It had served us well for years, but it appears that increasing $LN_2$ consumption got ahead of us on one long interval between fills and the detector warmed up - at least some. We got it cooled down again and tried it out. There were peaks where they were supposed to be, but there was also a lot of intensity tailing off to the low energy side. I suppose that the crystal broke during warm up and we were having difficulty collecting all the energy from the x-rays and it was a function of the relation between where they arrived and the fracture(s) in the crystal. We had to trash that crystal and replace it. Bottom line - I would not try warming up any detector without the express guarantee of the vendor that it won't be damaged in the process. Warren Straszheim <wesaia@iastate.edu> 16 Dec 2005

As a general comment, I have had Si detectors warm up now and then, never during actual use though, as I always check $LN_2$ levels before turning it on. I then re-cool the detector and don't turn the power on until the next day, just to be safe, and get normal functioning back. More pertinent to your observation of 100% dead time, one time after a warm-up and subsequent cooling down, I too saw the high dead time, due to very high noise count, as it turned out. Of course, I "flipped out" thinking the detector was damaged! But eventually I realized that the calibration of the energy axis had "slipped" to the very low energy side of the axis and I was picking up very high noise counts from that region of the energy axis of 1 to about 0.4 KeV. This very low energy region of the system is usually blocked out by the baseline setting for normal functioning. After new calibration of energy axis using copper (just nicking the edge of a copper grid with the e-beam to get Cu-*L* x-rays at low energy end and Cu-*K* x-rays at high energy end), using my system's calibration program, the noise went away and normal functioning was restored. So I suggest trying to recalibrate your energy axis. Maybe that will work for you too. Gib Ahlstrand <ahlst007@umn.edu> 17 Dec 2005

**EDS - Oil on detector window**

*Here's a problem for the New Year: A couple of months ago, I re-pumped in situ my chronically leaky PGT Be-window EDS detector, and ran 50 °C air into the Dewar while re-evacuating using the chamber vacuum via a hose from the sample introduction port of the JEOL 840 on which the detector is mounted. The re-evacuation worked well, in that the LN2 consumption is now back to normal, but after re-cooling and switching on the bias again, there was no appreciable low-energy response and no visible Cu L peak at all! I realized that grease from the O-ring sliding seal through which the tube of the detector enters the chamber had melted and run down onto the Be window. Cleaning of the window with Freon restored the Cu L intensity, and all was well, until it became obvious that the window was re-oiling again at a much faster rate than ever before. Because we do quantitative mineral analysis, I keep a good handle on the window condition, evidenced by both Cu L and Na K responses. I have been using this detector for about five years and not found it necessary to clean the window before, but now the Na response halves in a few weeks. Re-cleaning with Freon restores the response again. In an effort to fix this, I have changed the rotary pump oil - installed an alumina-pellet foreline trap - changed the diffusion pump oil (Santovac, but the old charge was still light-colored) but still the darned window is oiling up. I'm running out of ideas. It seems that either the chamber atmosphere has become markedly oilier than before, or the detector window is now running colder than before. My money is on the latter, as it seems too much of a coincidence for some change in the back-streaming performance of the 840 to have occurred at the same time as the re-pumping of the detector, but I can't see what might have happened. Anyone got any suggestions? Ritchie Sims <r.sims@auckland.ac.nz> 11 Jan 2006*

What has happened to you has happened to many before you! Once you reach the point of contaminating your window with rotary pump fluid, it will have contaminated the complete vacuum system. Two solutions: Either take everything apart and clean it – yes, all the vacuum lines and column liners. Or use a hair drier to bake the pumping lines to drive off the contamination as best as possible, as well as cleaning the parts you are easily able to reach. Many times in my service technician career I have been forced to take this route. It puts you out of action for a day or so whilst the system recovers but in the main it seems to be a cure. But if you do not tackle the foreline trap idea, it will happen all over again - our chemical test said it was rotary pump fluid by the way. Steve Chapman <protrain@emcourses.com> 11 Jan 2006

Many thanks for the replies and suggestions so far, however, please note that this is a new condition. For the past five years the oiling has not been a problem, and it has started to occur only since the re-pumping operation described below in tedious detail. There have been no changes to the system apart from the three steps taken, one at a time, in an attempt to fix the problem (changing the rotary pump oil; installing the foreline trap; and changing the diffusion pump oil), and they have made no difference. I think it would be too much of a coincidence for the cause of the oiling to be unrelated to the re-pumping operation, so I think that complex cures such

# NETNOTES

as installing $LN_2$ traps, plasma cleaning, complete stripping and cleaning, etc, do not address the question, which is "what has changed to accelerate the detector window oiling so greatly, and how can I get back to the previous state?" I had thought that perhaps a drop of melted grease might have dripped into the diffusion pump, and be decomposing in the Santovac, which propelled me to change the Santovac, but I now realize that this is very unlikely to be the case, as the diffusion pump is not directly beneath the sample chamber, and even if it were, such a drop of molten grease would not make it through the water-cooled baffle above the diffusion pump. Plus, of course, the fact that very thoroughly cleaning both of th diffusion pumps and both of their water-cooled baffles has made no difference at all to the problem. Incidentally, the Santovac, after five years' continuous operation, was only a pale straw color, boy, that stuff certainly is stable, isn't it? I feel fairly sure that there is a simple solution to my problem, waiting out there to be noticed, so please keep the suggestions coming. Ritchie Sims <r.sims@auckland.ac.nz> 11 Jan 2006

Does the snout of the EDS detector feel cool to the touch, when you vent the chamber? Use the back of your hand to test it. I had one detector that oiled very badly and it felt cool to the touch, so it acted as an oil trap for the chamber. I sent that detector to be completely rebuilt as a light element, high resolution detector and that problem has vanished, so it was a condition of the detector, not the SEM, since the SEM was not touched. It may be that the insulation between the liquid nitrogen or the cooled components and the snout of the detector has changed with your warm-up and re-pump. I am wondering if the copper braid that conducts the cool from the dewar to the nose of the detector has moved or is touching something. None of this helps you in any way. You can clean the Be-window detector with a trickle of pure ethanol or iso-propanol in the SEM, if you can get at it. Mary Mager <mager@interchange.ubc.ca> 11 Jan 2006

This is a long shot and you probably would have noticed the symptoms, but here goes: I had one 840 that kept being "burped" first thing in the morning, but not after that. The key to finding the problem was that the LEDs on the vacuum control all cycled correctly, but it took me a while (since it only happened once a day) to notice that the actual valve operation for V2 (at the base of the second diffusion pump) was delayed just long enough to burp the diffusion pumps. Turns out the solenoid valve that operated V2 had Apiezon in it (a lot) and after sitting overnight would stick for about 2 seconds, only milliseconds longer than the delay for V5 to rough the load lock. This problem had been intermittent for years before I took on the service contract, but things work fine, now. Perhaps you have a similar timing problem. Of course, the most obvious thing was that the diffusion pumps dumped and you don't seem to have had anything that severe happening. Another thought: when you cleaned the diffusion pumps, did you also remove and clean the ballast tank located between them? Perhaps it has become too contaminated and is now backstreaming since there is only one diffusion pump between it and the chamber. Do you have any additional gauging to monitor the actual pressure in the chamber? There might be some clues there, also. I'm thinking along the lines of a slightly leaky V4. One last thought: Is the water flowing in the correct direction? An accidental reversal puts warm water running through the water baffles and virtually always results in oil on the EDS detector. Best of luck, Ken Converse <kenconverse@qualityimages.biz> 12 Jan 2006

**AFM - Sample preparation**

*We seek help for the sample preparation for AFM analysis. We are trying to scan the suspended nanoparticles in a semi-contact mode. We made a smear on glass slide and observed but were unable to obtain the good pictures. Shrunali Kulkarni <aarti_harle@yahoo.co.in> 07 Dec 2005*

Spin-coating would be helpful. I used to spin coat magnetic nanoparticles onto newly cleaved mica substrate. The results were amazing; there almost were no big aggregates on the substrate. Alternatively, you may try direct deposition of your suspension on the substrate. However, in this case you have to remove excess solvent by rinsing it with the solvent used in your sample preparation. I successfully got individually distributed magnetic nanoparticles on mica, too. Susheng Tan <sstan33@yahoo.com> 08 Dec 2005

I would recommend you use freshly cleaved mica as a substrate in place of a glass slide. The topography of glass slides can be as large as several 10 nm if not some 100 nm. Probably you will not be able to distinguish your nanoparticles on such a substrate. Dr. Petra Wahlbring <petra.wahlbring@goodyear.com> 08 Dec 2005