

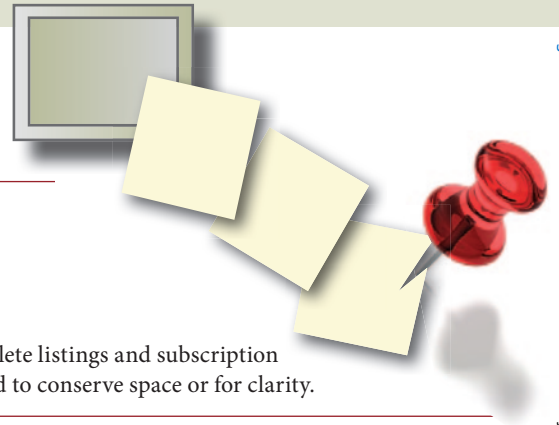
NetNotes

Edited by Thomas E. Phillips

University of Missouri

phillipst@missouri.edu

Selected postings from the Microscopy Listserv from May 1, 2010 to June 30, 2010. 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:

liver

*Thank you for those many that have tried to help with my problems with fixation and embedding of liver samples. There have been many good suggestions but so far none solves my problem. I would like to be a bit more specific in describing our protocol just in case someone might think of another possible cause. To recap, we have had consistent problems with extremely brittle blocks when cutting thick or ultrathin sections of liver. The tissue (not the surrounding blank Epon) fractures like glass, even during trimming with a double edge razor blade. We use the same protocol we use for the many other tissues that we embed well, so this problem is specific to liver. Our protocol includes primary phosphate buffered fixative (1.5% glutaraldehyde + 1.5% paraformaldehyde + 0.05% tannic acid) followed by 1% OsO₄ (with appropriate buffer rinses), dehydration in ethanol to 100%, a rinse in propylene oxide and infiltration and embedding in Spurr's resin, mixed according to Ellis. We have also embedded in Epon 812 substitute with similar brittleness. As I say, we use this protocol successfully for all other tissues. One suggestion received is that there is some water left in the sample which makes embedded tissue in Spurr's very brittle. I am sure our ethanol, propylene oxide and media is dry, but is it necessary to dehydrate liver longer than other tissues? The tissues are initially fixed in another laboratory and then sent to us. The tissue pieces are about 2 mm × 2 mm but were fixed in two changes of primary fixative for several hours each; then stored for a couple of weeks, then sent to us. Once in my laboratory, they are cut down so that one face is no larger than 0.5 mm. Then they are osmicated, dehydrated and embedded. Could either the initial excessive size of the tissues, excessive fixation time or storage in buffer be a factor? Does anyone have another suggestion? If you work successfully with liver, would you mind sending a protocol? **Doug Keene drk@shcc.org Mon May 3***

Well, I hesitated to respond, but when we see this problem in plant tissues, it's the embedding—resin infiltration, that's the problem, not fixation or dehydration. You may want to try infiltrating more slowly—with smaller resin increments, or for somewhat longer than whatever protocol you're using at present. In plants, the problem is almost impermeable cell wall (i.e. extracellular matrix) layers in the tissue that either prevent or greatly slow down infiltration of resin polymers. Are there any structures in liver that might slow polymer diffusion? In algae, for example, some ECM components are very impermeable, especially after osmication, and to avoid collapsing algal zygotes in some species (because solvent can diffuse out rapidly but resin can diffuse in only slowly), a colleague used to increment the resin by 2% per day over the beginning and end 10–16% steps of infiltration, with larger steps in the intermediate concentrations—it took a month to infiltrate. It did mean she had to do this only once and had perfect TEMs.... That's an extreme example, of course! **Rosemary White rosemary.white@csiro.au Mon May 3**

I also hesitated, knowing that it is unlikely that I might have any experience beyond that of yours or John's. Since you explained

more, though, this has moved into a mystery category, so ... (1) I do have some successful experience processing liver. It was ~15 years ago, but I remember the protocol firmly and also that there were no trouble with blocks. I studied mitochondria as an undergraduate, and so the ultrastructure was good too. 5% glutaraldehyde in 0.1 M sodium phosphate buffer for 2–3 hr. Bigger pieces were put in this fix and cut down to EM size by slicing with 2 sharp razor blades. After some short time, the sample was put in the refrigerator, and all steps from that point on done in the refrigerator, until acetone. 1% OsO₄ in same buffer. Buffer washes. 50% ethanol, 60%, then overnight in 70% with 1.5% uranyl acetate. Next day, 80%, 90%, 2x96% (that's what it said on the bottle, pure ethanol but not dried). Then 2x acetone, 15–30 min each at room temperature. Then 3:1, 1:1, 1:3 acetone:Epon (original Fluka Epon). Then the barbaric step of 12–24 h at 37°C in pure Epon, then 48 h at 60°C. The liver came out excellent. You can see that this protocol is not optimal in terms of infiltration, and it did get me in trouble with some other objects (like holes in bacteria), but not with liver. A higher percent of glutaraldehyde (4–5%) has always served me very well. For the kind of work I was doing then, I never saw any advantage adding formaldehyde to glutaraldehyde. Fine detail of the mitochondria always came out worse. (2) Doug, is that human liver? What I did was mouse and rat, of course. With humans, who knows what they'd been eating and drinking. (3) One thing that immediately caught my attention in your second post—that someone else was doing the fixation. Could it be something they do there? (4) Finally, if you can't solve it but still must trim and cut it, may I recommend my favorite blades? It is # 71930 from EMS catalog, Solingen Long Blades. Much harder and sharper than anything else. These will make a cleaner cut. **Vlad Speransky vladislav_speransky@nih.gov Tue May 4**

I had a similar problem last week with some nerve tissue. It was embedded in EMbed-812 (Epon alternative). However, the tissue was brittle and crumbled only in some areas and I am quite certain that it is related to poor fixation in those areas. I suggest that you participate in the fixation at least once and make sure that the tissues blocks are minced quickly and with minimal mechanical distortion to a size about 1 mm × 1 mm. The prolonged storage is not helpful but may be unavoidable. Let us know if you find the solution. **Larry Ackerman larry.ackerman@ucsf.edu Mon May 10**

Specimen Preparation:

silicon microspheres

I mostly work with biological samples. I have a researcher who is working with crystals formed from ~12 nm Si microspheres; this is somewhat like opal, I think. It forms nice crystals that are easily cleaved with a little pressure from forceps, etc. Lacking a field emission SEM, we have shadowed the surface of the crystals and find a nice hexagonal packing. They are a bit too small to be able to cleave in a controlled way in a chosen plane. The researcher would like to know if the crystals have



SO MUCH **MORE** THAN MICROSCOPY.

The College of Microscopy is now the



HOOKE COLLEGE[™]
OF APPLIED SCIENCES

Our name is not the only thing changing at **Hooke College of Applied Sciences**. We are expanding our offerings to include even more courses in microanalytical techniques and state-of-the-art instrumentation.

What is not changing is our commitment to expert-led, hands-on learning using modern instrumentation. We remain your leading resource for expanding skills and developing career opportunities.

Interested in increasing your scope? Learn more today.
630-887-7100 or **www.hookecollege.com**

UNLIMITED SCOPE in Training and Education

the same order inside as we see at the surface. I don't think it would make a crystal if it was not similarly ordered inside, but I guess one has to look. So they would like to cut sections to see an arbitrary surface. I embedded some crystals in hard epoxy and tried sectioning with a diamond knife; the crystal shatters and the section separates where the crystal was, leaving small shattered fragments clinging to the edges of the resin. I have to admit that I did not use my good diamond knife, but an older one that produces a few knife marks and is certainly not optimally sharp any longer—because I didn't know if it would damage the knife. The resin sectioned fine. I tried various cutting speeds, and thicknesses from 50 nm to 90 nm, all with the same outcome. The questions: Is it possible to section crystals in this way? Would this likely damage a "standard" biological diamond knife? Would FE-SEM of a cleaved crystal surface be a better approach? Is there another approach to take? **Dale Callahan** dac@research.umass.edu Tue May 4

No. You can't prepare Si specimens with a microtome. I wrote a short note on the subject in *Microscopy Today*, 14, 6, November 2006, pg 58, titled "Just Say NO to Microtoming Silicon!" that explains why you will wind up with a pile of Si dust and possibly damage your knife. You can download an MT Archive PDF at <http://www.microscopy-today.com/index.jsf;jsessionid=15B61AFA7C53C9E9CAEAE496FEF4BA5E>.

Polishing and etching your mounted specimens will work, but inasmuch as your particles will be randomly oriented, you can't count on seeing the "nice hexagonal packing" in all of the particles. The crystal lattice is present in all of them—it just won't be visible unless you find particles oriented in one of the principal crystallographic orientations. The <111> orientation for a hexagonal surface, etc. **Ron Anderson** randerson20@tampabay.rr.com Tue May 4

The small fragments that are attached to the sections might have the answer for you. Kind of "micro-cleaving" approach. Look at them in your TEM and evaluate the microstructure, near the sharp edges are the best locations to find thin (<200 nm for 100 KV instrument) areas. Also diffraction analysis with condensed beam (CBED) might yield some suggestions on the crystallinity of the structure. Other approach would be to use "mortal and pestle," then rinse in DI water and deposit on C-coated grid, then review in bio-TEM. **Jerzy Gazda** jerzy.gazda@ceriumlabs.com Tue May 4

I definitely second this idea. However be careful that most often the grid is turned upside down into the microscope and if the particles deposited on it do not firmly attach, they will contaminate the microscope. So take care to shake the grid a bit to get rid of the loosely attached particles before inserting the specimen holder. **Stephane Nizets** nizets2@yahoo.com Wed May 5

Specimen Preparation:

LR White vs Gold

Can anyone give me a comparison of LR White (LRW) and LR Gold (LRG)? **Tom Bargar** tbargar@unmc.edu Mon May 24

It depends on your application. LRG is UV polymerized; LRW is generally polymerized with heat. LRW works with osmicated tissue, LRG doesn't. It is a lot easier to get nice thin sections of LRG compared to UV polymerized Lowicryls. I like LRG for EM immunocytochemistry but if you want to do immuno-staining of 0.5 μm sections, nothing compares to BMMA. **Thomas E. Phillips** phillipst@missouri.edu Mon May 24

I used LRW and polymerized it with heat also. It came with a little packet of white powdered accelerator, but I didn't use it. It did polymerize with heat, I believe a 60°C oven. I have never used LRG because it was more expensive. **Barbara L. Plowman** bplowman@pacific.edu Tue May 25

Specimen Preparation:

UV and low temperature resin embedding

I am trying to polymerize LR White at -20°C (no success after 24 hours), in a Leica AFS unit. The UV system consists of 10 UV LEDs that are ~10 cm above the floor of the chamber. I can get the resin polymerizes in a 60°C oven in ~12 hours. If the problem is the LR White, can anyone advise me as to which resins work well with low temperature with UV polymerization? **Charlene Wilke** c-wilke@northwestern.edu Thu May 6

Is it possible that you somehow have confused LR White with LR Gold? LR Gold is the one people used to polymerize at -20°C (and get endless ribbons of sections out of it). Or Lowicryls, of course. Lowicryls on Leica AFS = tons of literature. **Vlad Speransky** vladislav_speransky@nih.gov Thu May 6

I am no LR White specialist, but I remember some past discussions about it. First of all you must make sure to avoid contact with oxygen in the air to allow polymerization. Second, you must make sure that UV light can penetrate your sample. **Stephane Nizets** nizets2@yahoo.com Fri May 7

Specimen Preparation:

freeze substitution

I have been performing high pressure freezing (HPF) and freeze-substitution (FS) on cell culture with vaccinia. Tried a number of FS cocktails in an attempt to determine the best ultrastructure freeze media. When I use 1% and 2% OsO₄ + 0.1% uranyl acetate in acetone, the mature virions are electron dense and I couldn't resolve fine detail. I recently tried 0.5% OsO₄ + 0.1% uranyl acetate in acetone with the anticipation that the mature virions would be less dense. Surprisingly, the virions had reversed contrast. The FS schedule was -90°C 3 days and gradually warmed to 21°C, acetone washed 4X and resin infiltrated. One suggestion was to use 1% OsO₄ + 0.1% uranyl acetate in acetone and acetone wash at -80°C instead of leaving in OsO₄ for the duration. Can anyone explain why concentration change of the OsO₄ resulted in negative contrast? Other cocktails used were 0.5% glutaraldehyde + 0.1% tannic acid in acetone, 2% uranyl acetate/methanol in acetone. Both resulted in patches of cytoplasm loss. **Karen Kelley** vau@ufl.edu Tue Jun 1

In an earlier life I did a ton of freeze substitution although never of viruses. My experience is that the osmication reaction is sensitive to subtle triggers at -80°C. Sometimes after FS in acetone, rinsing the now light brown tissues in 100% ethanol several times and then storing in ethanol overnight led to an intense osmication reaction with the tissue and ethanol solution going pitch black. I don't have a solution for you but you could try FS in tetrahydrofuran. We got startling different views with this solvent compared to acetone or ethanol. It might be worth a try. **Tom Phillips** phillipst@missouri.edu Tue Jun 1

Specimen Preparation:

osmium + ferrocyanide

I embedded routinely processed mouse brain in EmBed 812, no problems. The following week I embedded similar tissue processed the same except that I used an osmium ferrocyanide mix for osmication instead of plain buffered osmium. All other steps were identical. The Os-ferrocyanide brain is impossible to cut, acts like it was not infiltrated. Pure plastic cuts fine. Is it possible that Os-ferrocyanide reacts with something in the EmBed 812?? **Geoff McAuliffe** mcauliff@umdnj.edu Thu May 13

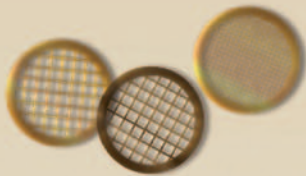
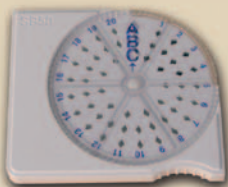
I have done 1000+ blocks of osmium + ferrocyanide fixed tissues into Epon-type resins without problem. I haven't used this combination in a while so I can remember whether I used it once the switch to Dpon 812 substitutes occurred but think I probably did. I doubt that is the problem. **Tom Phillips** phillipst@missouri.edu Thu May 13

SPI Supplies.

The complete source for all your microscopy needs...

just a click away.

2spi.com



Visit SPI Supplies to view the complete on-line catalog with up-to-the-minute product and pricing information.



SPI Supplies Division of **STRUCTURE PROBE, Inc.**

P.O. Box 656 • West Chester, PA 19381-0656 USA

Phone: 1-610-436-5400 • 1-800-2424-SPI (USA and Canada) • Fax: 1-610-436-5755 • E-mail: sales@2spi.com



You raised an interesting topic. Last month, I posted an entry reporting some infiltration problems using EmBed 812. I use a similar protocol for *Xenopus* tadpole eyes (which are approximately 1 mm³), involving osmicing the samples with potassium ferricyanide. As a result, the samples are (again) impossible to cut, although the ultrastructure seemed well preserved, indicating that there is a dehydration/ infiltration problem. I am currently modifying our standard protocol (basically increasing dehydration and infiltration times) and will consider removing the potassium ferricyanide, as I was already advised. **Tami Boga** tboga@interchange.ubc.ca Fri May 14

RE the query whether the FeCN might react with the resin, I can't help remembering discovery of the necessity for excluding NMA (MNA) from original Epon mix, using Epon DDSA (or Araldite-DDSA formula) instead, if one wishes to stain sections with permanganate rather than oxidize the embedding resin. (J Cell Biol 1965 26:309). **Mike Reedy** mike.reedy@cellbio.duke.edu Fri May 14

I have used permanganate as a fixative and stain and it is a whole different beast. The problem with permanganate is that it is such a strong oxidizing agent. Adding ferrocyanide to osmium reduces the oxidizing power of the osmium so should lower the risk that osmium would react with any resin component. Furthermore, the use of FeCN-Os is as a fixative so the tissue has been typically washed and dehydrated prior to exposure to the resin unlike the case where permanganate is used on sections. **Tom Phillips** phillipst@missouri.edu Thu May 13

I don't know if this works for animal tissues, or works as well as the OTO method, but a less toxic (yet equally tissue-blackening) protocol involves incorporating tannic acid into glutaraldehyde and/or formaldehyde fixative, then "developing" with ferric chloride. Alternatively, just adding tannic acid to the fixative before osmium staining can also enhance membranes. For an older review: Chaplin, AJ (1985) Tannic acid in histology: an historical perspective. Stain Technology 60: 219–231. Cheers, Dr **Rosemary White** rosemary.white@csiro.au Mon May 24

I agree tannic acid is a good approach. It is important to use the "right" type of tannic acid. I think it is the low molecular weight species but I would check this before proceeding. **Tom Phillips** phillipst@missouri.edu Mon May 24

Tom is right: it's important to use the monomeric kind, like Mallinckrodt 1764. **Phil Oshel** oshel1pe@cmich.edu Tue May 25

You can use tannic acid as a mordant to lead to enhance membranes stain. Simonesque and Simonesque, Journal of Cell Biology, Jan. 1976 is the reference. I don't know if you can still get EM grade tannic acid from Mallinckrodt or not. That's what you need. That's what the Simonesque's used. It will enhance membranes beautifully. Your fixation should be a standard glutaraldehyde fix. **Barbara L. Plowman** Bplowman@pacific.edu Tue May 25

So I took some more of the same brain tissue and used fresh osmium w/o ferrocyanide and I embedded 100 micron Vibratome sections in fresh Spurr (according to Anne Ellis). Same result! Badly fixed brains (by another lab) seems to be the problem, not a reaction between osmium+ferrocyanide and EmBed 812. **Geoff McAuliffe** mcauliff@umdj.edu Thu Jun 3

Specimen Preparation: enhancing membranes

I have a postdoc who would like to see the membranes of the vesicles she is interested in "stand out" better from the cytoplasm in the cells. She brought me an old paper where the fixation protocol pretty much extracted everything out the cells, except membranes—similar to the days of using potassium permanganate, etc. I would appreciate any and all advice on fixation protocols that may help to enhance the

visibility of membranes, while maintaining a good fixation of the cells without extraction of the cytoplasm, etc. **Tom Bargar** tbargar@unmc.edu Mon May 24

I have had good success with the OTO method—osmium thiocarbohydrazide osmium of Seligman et al. 1966. You won't believe how black your tissues turn! See Willingham and Rutherford 1984 J Histochem Cytochem 32:455–460 <http://www.jhc.org/cgi/reprint/32/4/455.pdf> for a good start. **Tom Phillips** phillipst@missouri.edu Mon May 24

We use reduced osmium as a secondary fixative for membranes. Works especially well on cultured cells. **Pat Kysar** pekysar@ucdavis.edu Mon May 24

Specimen Preparation: stain variability on LM sections

I don't do a lot of semi-thin sectioning, but a recent request has led me to the following question: Why would two (different animals) of the same thick (0.25 μm) semi-thin sections show different color hues when stained identically? The stain is Richardson's (1% methylene blue, 1% azure II, 1% borate) stained for 30 sec on a hot plate then water rinsed and sealed with Permount. One of the two sample sets shows more purple than blue? Any thoughts will be greatly appreciated.

M Delannoy delannoy@jhmi.edu Mon May 17

My first comment is that 0.25 μm is pretty thin for a semi-thin. I usually use 0.5 μm. Unless you are carefully selecting sections by their interference colors while floating on water, I doubt they are all uniformly 0.25 μm and this might alter staining patterns. In addition, the degree of cross-linking of the resin will alter the staining properties so if there are different batches of plastic resin used for embedding or they were heated down in the oven at different shelf levels and the top of the oven was hotter, you may get differences in staining. 30 sec seems short for staining—I might go longer at a lower temperature to get a more even end result but my experience would suggest it is subtle differences in the resin that caused the difference. Differences in fixation duration might also impact staining. **Tom Phillips** phillipst@missouri.edu Mon May 17

One reason is that methylene blue oxidizes in solution to a mixture of azure A, azure B, and other thiazine dyes. The mixture is uncontrolled, but this is done deliberately for some polychrome staining. What are the differences between your samples? Species, tissue, . . . ? If just different individuals, and all else is the same, I'd look to section thickness and subtleties of embedding, fixation and the like. **Philip Oshel** oshel1pe@cmich.edu Mon May 17

Thanks to all of you who responded to my question so quickly, I received many helpful insights and suggestions. I would like to summarize for you the responses. 1. Methylene Blue oxidizes in a mix of Azure A and B and other thiazine dyes. It is uncontrolled as it occurs over time. 2. pH differences of the stain, if the sections are of appreciable volumes, pH differences of the two sections may have altered the pH of the stain. 3. Differences in pre and post staining drying times and position on the hot plate. 4. Permount contains organic solvents which elute basic stains from sections. I overcame everything by matching the interference colors, staining as soon as the sections dried, (30 sec staining), rinse, dry and controlled position on the hot plate. I mounted immediately in Permount, and then recorded the images immediately. This seemed to work perfectly. **Michael Delannoy** delannoy@jhmi.edu Wed May 19

Specimen Preparation: picric acid

I am trying to find information about the chemistry of picric acid fixation. Specifically, I want to know what functional groups are cross

Microscopy Society of America Awards

Nominations are now open for the Microscopy Society of America Annual Awards. The awards process is one way in which the Microscopy Society of America recognizes the significant and diverse contributions that individuals make to our field. Deserving nominations for consideration should be submitted online no later than December 15th, 2010, to:

AssociationManagement@microscopy.org

The Main Society Awards Are

Distinguished Scientist Awards

These Awards recognize preeminent senior scientists from both the Biological and Physical disciplines who have a long-standing record of achievement during their career in the field of microscopy or microanalysis.

Burton Medal

The Burton Medal was initiated to honor the distinguished contributions to the field of microscopy and microanalysis of a scientist who is less than 40 years of age on January 1st of the award year. (Please note the change in the selection criterion regarding age.)

Outstanding Technologist Awards

These Awards honor technologists from both the Biological (Hildegard H. Crowley Award) and Physical Sciences (Chuck Fiori Award) who have made significant contributions such as the development of new techniques which have contributed to the advancement of microscopy and microanalysis.

Morton D. Maser Distinguished Service Award

This Award was initiated to recognize outstanding volunteer service to the Society as exemplified by Mort Maser, who served the Society for many years with great dedication. This award is made to honor an MSA member who has provided significant volunteer service to the Society over a period of years.

**Further details of the nomination process
can be found on the society webpage at:
www.microscopy.org**



linked and what the pH dependence of the reaction is. Any ideas on where I might find this? **Dave Knecht david.knecht@uconn.edu Thu May 27**

One good source for this sort of information is “Histological & Histochemical Methods: Theory & Practice” by J.A. Kiernan. Kiernan states that picric acid is a coagulating fixative that works by binding with basic groups of proteins, and precipitating them. pH 1.5–2. The precipitation is reversed if the solution pH is neutralized. **Philip Oshel oshel1pe@cmich.edu Thu May 27**

Specimen Preparation: oxidized grids

I am using up a large stock of inherited grids from various vendors and discovered that many of the copper grids develop a dark black surface after coating with a support film. I have used Butvar and Ultem (polyetherimide also known as PEI) with chloroform as the solvent. I tried lightly etching with 1N HCl and always clean with ethanol. The “oxidation” continues and after a month or so the grids have “hairy” edges and the films are contaminated. I have never seen this previously and it has not happened with nickel grids. Any suggestions for eliminating the “oxidation?” **Larry Ackerman larry.ackerman@ucsf.edu Thu Jun 10**

A soak in some dilute orthophosphoric acid will generally clean up tarnished copper. A final rinse with ethanol should then see them clean and ready to go. Regards, **Dave Mitchell drg.mitchell@sydney.edu.au Thu Jun 10**

Specimen Preparation: AuPd v. Pt grain size

In regards to sputter coating ... will someone please inform me if anyone has ever compared the grain size of Pt to AuPd, particularly in terms of high resolution SEM analysis? **Marissa Libbee mlibbee@gmail.com Tue Jun 29**

I presented on this very topic at SCANNING in 2006. The Pt grain size is almost undetectable at 2 kV, 300,000× magnification in a Hitachi S-5200 FE-SEM. AuPd (as is Au) is very noticeable under these conditions. Pt, Cr, and Ir all have about the same surface quality. In my notes I have the approximate grain size of AuPd to be around 1.8 nm and Pt to be around 1.5 nm using the conditions in my Emitech K575X sputter coater. Sputter coating variables will have an effect on the grain sizes of the metals. **Becky Holdford r-holdford@ti.com Tue Jun 29**

A seemingly simple question but a not simple answer. Au coating is like Spiderman. Au/Pd is good but not as good at Pt, Pd or Ir. But the big variable is the terminal vacuum. Poor vacuum (30 mT) is not going to be able to put down an invisible coating that would or could be done at 15 mT. That is just vacuum. Longer coating time at lower current will improve the coating. At 200,000–300,000× magnification on a Zeiss FESEM I can see the Au/Pd coating. Bad. Not so with Pd or Ir at 15 mT. But I do find that it takes a longer coating to achieve a good result. This is using a Denton Desk IV TSC turbo coater with tilt and rotate. In conclusion, I would prefer the Pt over Au/Pd but search for Pd or Ir in its replacement. Recovery in Las Vegas has all of these targets. Good prices. **Gary Gaugler gary@gaugler.com Tue Jun 29**

Microtomy:

thin sectioning of fruits, vegetables, seeds

I am new to the world of thin sectioning plants (fruits, vegetables, etc) for examination with light microscopy. I would like to know what is the difference between an ultramicrotome and a cryostat and when would you choose one over the other. Does anyone know of training that is offered to help me get started in this area? **Nancy Hirdt nancy.hirdt@pepsico.com Wed May 5**

There are a number of alternatives. One thing to consider for seeds is the confocal microscope. They optically section so that you can “look inside” without having to do any cutting. We’ve also used this technique successfully on things like pollen. As for the more conventional embedding and sectioning of larger items like fruits and veggies... I’ll leave that to the sample prep gurus on the listserver. **Barbara Foster bfoster@mme1.com Wed May 5**

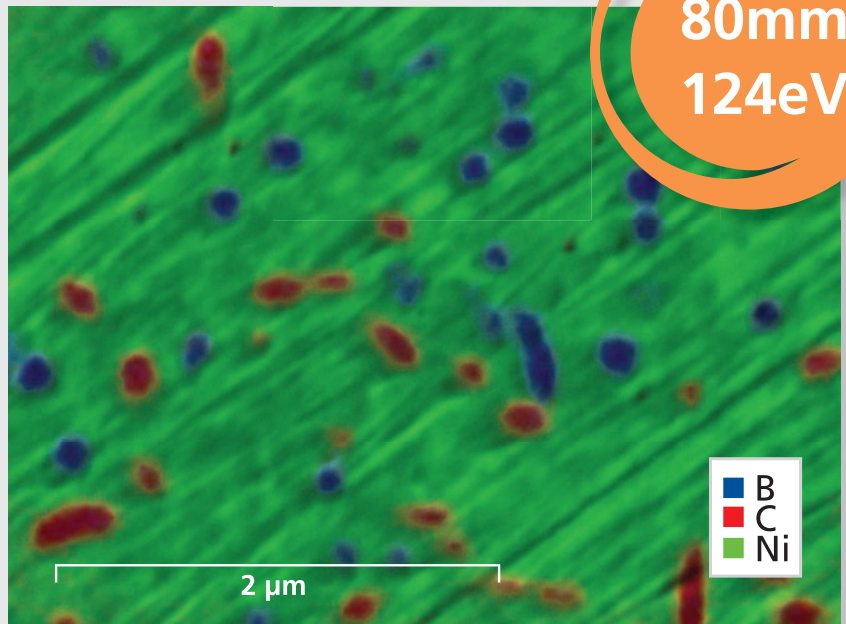
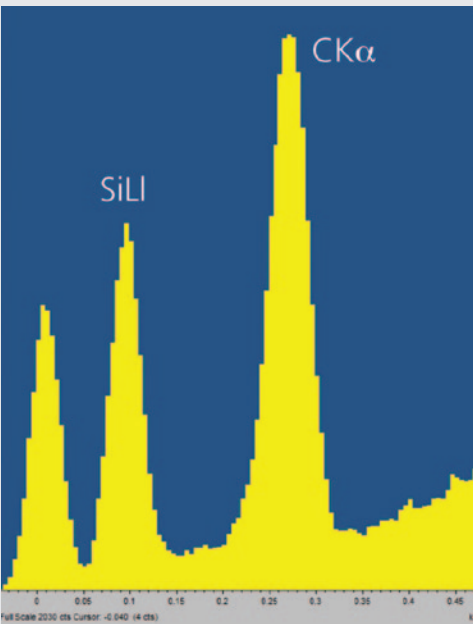
I would start first with hand sections using a razor blade. If you want to look at plants in something close to the living state, this is the way to go. Most of the work we do here requires no more than this, if pushed we fix and section—some cryostat sectioning, other resin sectioning, if pushed further we go to electron microscopy. A very good place to start is a recently published (2008) spiral-bound book “Teaching Plant Anatomy” by RL Peterson, CA Peterson and LH Melville, NRC Press, ISBN 978-0-660-19798-2. It’s really terrific, it gives detailed step-by-step instructions and it’s all hand sectioning, staining if required, mostly simple stains, and observing with light or fluorescence microscopy, with some macro work under the dissector. You can look at fruits and seeds this way, I’ve hand-sectioned dry barley grains and briefly brushed the cut surface with stain and imaged under the fluorescence dissector. We’ve also looked at grapes and oranges, and at the small end I’ve even hand-sectioned arabidopsis anthers (a sapphire dissecting knife is useful here). For hard or large structures, you could investigate a sledge microtome, which people have used here to section woody roots, hard tobacco stems, bits of trees, etc. The only error in this book is that the microscope setup on p. 4—“raise and lower the condenser until the most even illumination is achieved” is not correct, you need to adjust for Koehler illumination. The book below and numerous websites do a better job telling you how to set up a microscope. If you really have to, for embedded plant tissues, try “Plant Microtechnique and Microscopy” by SE Ruzin, Oxford University Press, ISBN 0-19-508956-1. **Rosemary White rosemary.white@csiro.au Thu May 6**

Digital Imaging

25 years ago the idea of digital image capture was totally enamoring. The ability to get rapid prints of clinical and diagnostic material was fantastic. Even if the prints from the original fiber optic systems were not to the same resolution, the improvement in time to report from almost a week to less than a day was fantastic. And there was always wet chemistry for publication prints or if we really needed resolution. 25 years later, as we finally get digital, I find the reality somewhat less than captivating. But then, I have 40 years of wet chemistry experience, and at the risk of sounding conceited, I am fairly good at it. The problem is—we have had a used Gatan system installed, and are capturing the images using Gatan DigitalMicrograph software. On the monitor the images look alright, but just that—alright. Frankly, the resolution is less than on the focusing screen, but for diagnostic, they are alright. Hopefully I will be able to figure out how to actually adjust the different brightness/contrast settings so that we can get away from ‘averaged’ optimized data capture, which should improve the original data. The system falls apart when it is time to take the micrographs away to process and print them. I have been using PhotoShop CS4 Enhanced, with 64 bit processing on my computer at home, and am not all that impressed. Neither does Illustrator CS4 excite me. Ignoring the fine detail resolution—there is no such thing as a fiber, forget it—the immediate technical problem is getting acceptable prints. The adjustment of contrast and brightness seems to be highly limited before bloom effect takes over. There simply is not sufficient gray scale variance to get a good micrograph. The contrast is too extreme; the background is whited out due to saturation, etc. There is going to be a need to upgrade the computer and printer hardware and software.

X-Max: the biggest just got better!

This innovative next generation X-Max detector delivers the ultimate performance for large area SDDs with up to 80mm² active area



80mm²
124eV

ULTIMATE SENSITIVITY FOR NANOANALYSIS

New Standard resolution of 127eV Mn and 56eV C
New Premium resolution of 124eV Mn and 48eV C

- Most sensitive detector for light elements
- Widest solid angle without any compromise in SEM performance
- Unparalleled and accurate quant at both low and high count rates (1,000-200,000cps)
- Market leader in overlapping peak characterization and AutoID capability
- All resolution specifications guaranteed on SEM

To find out more, please email: nanoanalysis@oxinst.com
www.oxford-instruments.com/xmaxnew



The Business of Science®



*And I really want to do this right the first time. Which leads to the questions * What programs are people using that provide for better micrograph processing, * What printers are good. Frankly, the best we have here is not much improved over my very old HP 1200 toner printer in the lab. Again, as with processing the images, the best I can get is on my home system, where I use an HP6400 multifunction system. And I don't like those prints. So, thanks in advance. Paul R. Hazelton paul_hazelton@umanitoba.ca Fri May 14*

I'd like to respond to the recent posting by Paul Hazelton. However, I would like to first put out a disclaimer: As a person who has been involved with digital imaging on TEMs and SEMs for a while, and who is currently selling these systems, I am definitely biased towards digital imaging systems. Now, Paul brings up a number of good points. Perhaps I can add some light on this from another direction. There are many issues that have an effect on the "quality" of images, and I think it's instructive to look at some of them. Let's start by looking at resolution, as it is often stated that "film has a higher resolution." It turns out, in fact, that it is true. If you look at the MTF of film compared to that of, for example, P47 (scintillator material), which has been measured (references available, please ask), we find that film has around twice the resolution (50% point of the MTF) than the scintillator has. But the ultimate limit of the entire system is of course determined by the resolution of the TEM. John Spence measured the MTF of SO-163 and came up with about 32 lp/mm, or 30 micron, which also depends on the development procedure. That means that if you have a TEM with 0.2 nm resolution, you need a magnification of at least 150 k \times to see the detail on film. For digital, you need to go to something like 300k \times , but then you see the same detail. You lose some field of view, but you can resolve whatever the microscope can. So, resolution is clearly not the issue. Well, then it must be the dynamic range. But that's not it either. Our eyes have a dynamic range of maybe around 7 bits (we can distinguish perhaps 100 levels of grey). Film can have a different dynamic range, depending on the development process, but typically that would be 6–12 bit (64–4000 levels of grey, the latter only with significant reduction in resolution). Digital cameras can have resolutions between 12 and 16 bit, depending on various factors such as cooling, etc. Now, everybody would agree that 64,000 levels of grey are better than 200. So why do some people see digital images as inferior? I think the answer lies somewhere else. First of all, most people don't use the full dynamic range of their cameras, and it may not always be possible. The information may be "bunched" in a small range of grey levels, and then this information is "stretched" and "shifted" to make it visible to the eye. In extreme cases, one could wind up with only 50 levels of the 64,000 used, which would then be visible in areas that have fine grey level details. So, look at the histogram when you acquire images. If the data is all bunched up at one end of the histogram, the images will probably not look good. For example, if the background is all saturated, it is probably a good idea to reduce the beam current or exposure time. Then we have the linearity of the digital cameras. As opposed to film, they are very linear, which is a good thing, with a sharp cut-off at the low and high intensities. Film, in contrast, has an S-shaped response curve (gamma). This makes for pictures that are easier on the eyes, as the sharp cut-offs at saturation are absent, but makes film much less useful as an analytical tool. Of course, you can mimic that with a digital camera, as we can usually set the display to various gamma values. The last thing I think contributes is the fact that digital images are usually observed on a monitor and are very easy to manipulate. We CAN go to the extremes and beyond by simply clicking a button. We CAN magnify the image on the screen to ridiculous proportions just by selecting it with the mouse. That's not possible with film. You have to go back to the dark room and play with chemicals for an hour. Because of that we have all

learned that we can't blow up a negative too much, and so we never see the effects. Film definitely has an advantage in terms of information density. It can record more information over a larger area. But digital has the advantage in almost anything else, and the film's advantage can be overcome with things like taking serial images and mounting them. It takes a bit longer, but serves the same purpose. Now for Paul's questions. I think that Photoshop and Illustrator are fine products, but they are designed for color photos with the aim of publishing them. Photoshop alone, for example, does not even have the concept of magnification, unless you use some plug-ins. I am not so familiar with Illustrator, but I guess it's the same story (this may be a good point to remind everybody that I am earning money from selling a competing product and you should come to your own conclusions). So, the first thing I would suggest is to look at software that is made for microscopy, and go from there. And you don't have to stick with what we are selling; there are many fine products out there. As far as the printer goes, it is a developing story. My suggestion would be to look for a high quality ink jet printer and use high quality paper (photo quality) for the best results. Get a good, but inexpensive laser printer for the routine pictures. I have had good results with an Epson R800 Ink-jet printer (I think they don't make them anymore). Mike Bode mike.bode@resaltatech.com Fri May 14

I'm with you guys; I miss the beauty of darkroom photography. I could carry on at length, especially about digital image manipulation. The *short* answer to your immediate question is, using Photoshop, go to Image - Adjustments - Levels (the first thing in the picklist for a reason). See histogram. Adjust the white triangle and the black triangle toward the edges of the histogram, and then move the gray triangle in the middle to where you like the results, usually more toward the peak. If you still want more contrast *after* this, *then* use Contrast and Brightness. This is the best thing you can immediately do, plus it's just about all you can do according to the MSA guidelines on the ethics of digital imaging, which I can't find on the new, redesigned MSA site. Tina (Weatherby) Carvalho tina@pbr.c.hawaii.edu Fri May 14

I might add a bit more to what Tina suggests. And I also understand and appreciate the fine points of wet photography. I have a very good 4K CCD camera on one of our TEMs and I rarely use it. On the other hand I have not printed in the darkroom for about 5 years and our last Durst enlarger is on its way to salvage (let me know if you want it). I capture images on film and then we scan the film at appropriate dpi for its intended use (usually 600 dpi for most images). I find I can capture better grayscale images on film faster than if I use the digital camera and it is much easier to manipulate the contrast gamma, and brightness when scanning to end up with a very reasonable digital image. However, I also can easily stigmatize and focus on the viewing screen and that is also almost a lost art. The 15 min it takes to develop 30–40 negatives is a minor inconvenience for the larger and higher quality viewing area. Yes, we then have to put in time scanning but the final outcome is worth the little extra time. I suggest you use Photoshop and use "Layer >>> adjustment layer >>> levels" rather than "image >>> adjustment >>> levels." Using the new adjustment layer lets you modify the original image and then save the image. This does not alter the original scanned image but rather saves the changes in a new layer. Then when you next open the image you can easily readjust, again without changing the original image. When you finally get the image you want for a specific purpose you can just flatten the image and do a "save as" to save the modified image. You still have the original unmodified image to archive. Debby Sherman dsherman@purdue.edu Fri May 14

You did not state what sort of "used Gatan system" you have, and that may be your fundamental problem. If you have one of the

older 1k × 1k cameras with 14 bit depth, the images are going to look disappointing no matter what software, computer, or printer you use.

John Mardinly john.mardinly@wdc.com Mon May 17

I recall an interesting discussion recently with a curator of photography at a well known museum here. She pointed out that the physical nature of a photograph is quite different from a print. In a print, the ink is deposited in a single layer on the surface of the paper. In photography, both in the film and the paper, the silver is embedded in a deeper matrix in the paper. The end result may very well be that “breath-catching” moment when looking at a photographic micrograph, since the image is formed by a combination of simple reflection and interference within the matrix. So, beauty is a little more than skin deep. **Joel B. Sheffield joelsheffield@gmail.com Mon May 17**

Adjusting the brightness and contrast is not enough. I have been doing fine art photography and showing my work in galleries and Photoshop is the program to use regardless if you are doing color or B&W. I don't print much of my scientific stuff anymore since papers are submitted electronically but after Friday's discussion I sent one of my DM3s (converted to 16 bit grayscale tiff) to my home machine where I have my Epson 7800, a 24 inch carriage archival pigment inkjet. The print I made from our 2K Gatan camera looked as good, if not better, than any of my silver gelatin prints of the past. I purchased my first high-end printer (Epson 2200) about five years ago. I was disappointed with the results because they weren't as good as prints from an older printer I had. I then took a course on digital printing and I took another more extensive course a few years ago. Photoshop does a good job of color management that a number of other software programs don't do (PowerPoint for example). My Epson 7800 has eight different inks (3 blacks if I remember correctly). A new Canon I saw advertised in one of my photo rags had 12 inks. Standard CMYK (4 inks) just doesn't do it because the blacks usually come out with a color cast. To get a good print you need to match the printer, the inks and the paper. This is done with a color profile file that is usually available on the paper manufacturer's web site. You also need to have a color calibrated monitor and work in a room with subdued light. One of the newer Epson printers is the Epson 3880 and it runs around \$1500. I keep mentioning Epson because they had the market until a few years ago and I haven't kept up with the other brands. These are inkjets and they need to be babied otherwise you will have problems but they produce fantastic results. I have a book on printing that I have read cover to cover. It is Color Confidence: The Digital Photographer's Guide to Color Management by Tim Grey. Now that says color but the concepts are similar. I have absolutely no desire to go back into a chemical printing darkroom. **Norm Olson nholson@ucsd.edu Mon May 17**

First: Digital Micrograph is a great program but not for printing. For printing you want Photoshop. But you can use almost any version since Photoshop 5. The trick is simply to understand how to print and what to print on. Best B&W will be Epson for so many reasons and three major patents that it isn't worth talking about. All pro photographers use Epson printers. We have tested printers for years now and the Epsos are clearly superior. The best current model is the Epson 2880. It will print up to 13" × 17" which covers everything except posters (We use an Epson 9600) The K3 (3 blacks are important because you can print at higher densities than 300 ppi. This is because the dot pattern can be denser if you have gray inks. Gray inks are very important for the best but you can do very well with a C88 plus on good paper. The C88 plus printer costs \$85. The 2880 is about \$800. The C88 plus prints the best images on plain paper. We have always compared our prints (and still do) to the same negative printed on Agfa Brovira paper. If you have enough pixels then the prints will be very close. The photograph will be sharper but only to a trained observer.

Now I have heard all the arguments about resolution and pixel size but for a biologist there is an important and rather simple calculation that I think defines the problem. The old photo prints were 8" by 10". A good digital print is about 600 ppi. The simple math says 4800 × 6000 pixels. That is going to take a rather large montage to equal with a smaller digital camera. So you either need a larger format camera, a large number of montage images or record on film and scan. 4k cameras are out of my laboratory's budget but a \$700 scanner can do 2000 ppi (we have calibrated it) Epson wouldn't you know. (D750-M pro or V700; also tested 4880 and 4990) It is quite easy to get enough pixels by scanning at 1200 ppi and then we are totally digital. When you want the best you can go up to 6000 × 8000 pixels. in one pass! So the procedure we follow is as Tina said: Levels Adjust histogram till black arrow and white arrow just touch. Then print. adjust gamma by at least 1.2 print again repeat until it is clear you have too much gamma. Why? Because gamma is like focus you don't know that you have too much until you go past it. After you have chosen the best gamma value you do a final histogram stretch to reclaim the contrast. We do this selectively in the dark region of the histogram where there is little data that will be masked out (lost). We can reclaim a huge amount of contrast while binning less than a couple of percent of the data The best histogram to start with is one that has no values in the dark or white regions (about 15–20 values) this insures the image is neither over or under saturated. When scanning, we scan in 16 bit mode then do a histogram stretch in 16 bit mode in Photoshop before converting to 8 bit mode for gamma adjustment and printing. Note no print driver known can handle native 16 bit images. Then proceed with Levels adjustment as outlined. With a good image, we can get publishable quality prints on any Epson on any paper. Secondly, you are not doing anything that is not scientifically correct. **John M. Mackenzie john_mackenzie@ncsu.edu Mon May 17**

When moving the white and black sliders in the Levels box within Photoshop, it is sometimes difficult to see where the histogram ends because so few pixels make up the darkest and brightest parts of the image (on the other hand, the Photoshop-mimicking free program, GIMP, provides a log scale histogram so that every pixel tone can be seen). Here's a tip: if you want to see the parts of the image with the darkest and brightest pixels, hold down the alt key (PC) or the option key (Mac) while moving the sliders. For the black slider, the image will turn white the moment you move the slider; for the white slider, the image will turn black. Move the slider until significant parts of the image appear, then back off until these disappear. Note the word “significant:” some parts of the image may be detritus or artifact. When these parts of the image appear, it tells you that a move of the slider any closer to the center of the histogram will result in setting the darkest and whitest parts of the image outside the dynamic range of the image (clipped or saturated values), which is okay for known, non-significant parts of the image, but not okay for significant parts as it will block detail. No one has addressed the issue of sharpening. I've noticed that some journals discourage sharpening, but since this thread speaks to printing to hard copy, sharpening may be an option for bringing out local contrast to better reveal details. Some call this “enhancement” or “doctoring” while others call it “resolution.” My thought is that any improvement in revealing detail is something that should be pursued: over-sharpening, or sharpening to the point at which artifact is confused with biology/materials must be avoided. A way to provide local contrast consists in duplicating the background layer in Photoshop to make a layer above the first layer (Layer > Duplicate Layer). Then use the High Pass filter (Filter > High Pass) and set the level between 1 and 4 to the point at which edges are visible but not so much the features. Set the Layer mode (in the Layers palette) to Hard Light and the image is sharpened. By clicking

on the eye icon (in the Layers palette) to turn off and on the layer, one can see the subtle effect of sharpening and consequent improvement in local contrast. This effect works much like selenium toning on photographs to improve acutance. Sharpening should be done before setting tonal levels, and to a duplicated image, not the original or raw image. Finally, in my own experience, the blackest tones on photographic paper exceeds that of inkjets, and the darkness of the black tones has a marked effect on the perception of contrast. Thus the necessity for more than one shade of black ink in an inkjet printer, as John had mentioned, and the necessity for sending out to an agency as mentioned in an earlier post. At one time the U of Minnesota core facility owned a Fuji Pictography 3000, which is a dye-sublimation/photographic processing device. This could achieve nearly the black tones provided on Kodak RC paper, and side-by-side comparisons with photographic paper could, in some instances, be perceived as “better” than the wet-processed papers, depending on the sample. A means for providing a greater perception of contrast can also be done by blue-shifting the image. As microscopists, we develop a “color memory.” Even though photographic paper is “black and white,” the Agfa papers tended to be bluer than the Kodak papers (which looked more brown under artificial light), and we became not only accustomed to seeing that color shift, but became judges of quality based upon the color. Try it sometime: make the image into RGB Color, and then use the gamma slider in Levels to adjust the blue channel, and then print.

Jerry Sedgewick sedge001@umn.edu Tue May 18

I have been enjoying the discussion about digital and film, but I want to make comment here: Digital image acquisition is no silver bullet. You cannot recover digitally what you don't have physically. To have a good digital image, one needs to start out with a good physical signal. While digital imaging can help with difficult situations and make a small contrast more visible, one has to have a contrast to begin with. What I want to say is that digital imaging does not absolve the TEM user from knowing the instrument and its limitations and capabilities. **Mike Bode** mike.bode@resaltatech.com Tue May 25

Image Processing:

deconvolution & confocal microscopy

I am a first-year graduate student trying to get started with co-localization analysis of IF images taken by confocal microscopy. I am working with fixed cells and mainly trying to measure co-localization of 2 proteins. My main questions are concerning the use of z-stacks and deconvolution: 1. Are z-stacks always necessary for measurement of co-localization, or are single images sufficient? 2. I understand that deconvolution is essential for wide-field images, but does it improve results that much when you're already using confocal? 3. If you would recommend deconvolution, is the ImageJ plug-in a good one to use? Thank you for your time & help. I have read many articles on this topic, but I keep ending up with conflicting answers. **Molly Shaw** mshaw1@lumc.edu

Scientists have published co-localization results using single planes from confocal microscopes, z-series, z-series with deconvolution and results from widefield microscopes with thinly sliced specimens. This alone creates confusion about the “right” method. Do yourself a favor and go through the effort of deconvolving images. You will then have a greater likelihood of being published in a high profile journal, although you might be asked for more robust methods like Fluorescence Lifetime Imaging Microscopy (FLIM) or Forster Resonance Energy Transfer (FRET). Confocal microscopes do collect out of focus fluorescence, and there is elongation in the z-axis, and deconvolving images ameliorates those problems. If nothing else, do the following: Use a high N.A. lens (1.4 or better). Mount the specimen in a medium that has a refractive index of 1.4 or better. Use a coverslip

thickness intended for the oil objective (0.17 mm). Make sure you don't just place one image over another (red + green = yellow—presto! Co-localization!); show results using scatterplots. If the feature you are labeling is at near subresolution dimensions, then use Nyquist rates for collecting (your core microscopy lab can provide info about Nyquist rates for their equipment, or at least a way to determine these). I have tried Volume J a few years back and it crashed with large image sets. I suspect computers/software have caught up by now. Certainly Image J/Volume J is good for attempting deconvolution. It's advisable to make a point spread function (psf) from subresolution beads (0.8 microns, or what you read in the literature as the bead to use for the objective: I can't remember offhand what I used way back when) and then deconvolve using the psf. Have fun! **Jerry Sedgewick** sedge001@umn.edu Tue Jun 8

You are right that a confocal will not benefit from deconvolution software anywhere near as much as a widefield microscope, for the reason you suggest, i.e. much of the out-of-focus information is removed by the confocal iris/pinhole in any case. I use Improvison's [Perkin Elmer] 3D Volocity software to look at things like 3D images, 3D cellular volumes and cell morphometry. In order to get the best from a Z slice you are advised to over sample the z-stack, i.e. for a 0.9 μm optical slice capture the z stack at least 0.45 μm apart. Our Zeiss 510 has a button to automatically 'double oversample' like this. For the likes of Volocity deconvolution, Improvison recommended to me that you massively oversample the confocal z-stack with ten or more slices to every 0.9 μm z focus step. I think the rep suggested perhaps up to as high as 100 \times oversampling. I rarely use the optional 'Restoration' module [deconvolution] Volocity module as I only use the confocal for z-stack, and double oversampling and 3D reconstruction alone with Volocity is adequate for my needs. For co-localization I would initially try and tackle the co-localization quantification in 2D using MetaMorph probably just because it's just easier. I don't use ImageJ very much as I have a MetaMorph license, and we don't have the 3D Quantitation module for Volocity in any case, that might be needed for 3D co-localization quantification—if interested just ask Improvison about that as their support is superb. Always be suspicious that any co-localization isn't bleedthrough. You can check this via spectral un-mixing on a 510 Metahead or just try imaging single labeled samples of each fluorochrome and check there's no signal in the other channels where there is now no longer any label at the same confocal imaging settings. Don't forget a sample with no label just in case of autofluorescence. To quote Improvison's own website: “If you acquire your images from a widefield microscope and want to volume render or make measurements from the image data, you will need to use an image restoration technique [de-convolution] to remove the out-of-focus information—a product of the optical properties of the microscope. Even if your images are captured using a laser scanning or spinning disk confocal microscope, the image quality may benefit from image restoration.” The word ‘may’ is probably significant. **Keith J. Morris** kjmorris@well.ox.ac.uk Thu Jun 10

Be very careful with quantification in fluorescence. A higher signal doesn't necessarily mean a higher concentration of the antigen, it may also mean a better antibody, a better accessibility for the antibody, a better stability from the laser light and so many other things. You could trace a line through the structure and show a profile scan (confocals are good at that), this is a nice way to concentrate the attention only on one structure and demonstrate the co-localization of 2 peaks of intensity. The profile also allows a fine analysis of the both signals intensities and who knows, perhaps you'll discover that both signals are not just perfectly superimposed, but are next to each other (very close), or that one signal has more a ring pattern and so on. Small differences in intensities are not easily

appreciated with eyes especially at high intensities, but when they are presented in histograms it is much easier to see. It may be that both signals cannot be collected at a similar intensity as to observe a nice mix of both like a nice yellow from red and green mixing. For profile scan it doesn't matter because you just want to show that the position of each peak is similar, whatever the height of the peaks. But for mixed images it is of course better if the structure has a well balanced mix of both signals. Say the red is much stronger than the green, the merged image shows a red structure; well it is not a very convincing demonstration of co-localization. Thus it is often required to increase the intensity of one signal. Take care to increase the overall intensity of the whole picture and not only part of the histogram like playing with the contrast or even worse: only a part of the image since this is a misconduct. If playing with the contrast is acceptable, do not forget to state it but I would avoid it. **Stephane Nizets nizets2@yahoo.com** Fri Jun 11

TEM: filament life

*I work with two Philips CM series TEMs. I am trying to help diagnose some filament life issues, and I need to know what the temperature I am heating the filament to is, in a real unit (kelvin), instead of just knowing it is heating stage 21. Does anyone know of a conversion procedure? The same question for the vacuum readings, the emission current scale, and is it possible to give meaningful units to what the emission level setting? **Ben Micklem ben.micklem@pharm.ox.ac.uk*** Wed May 2

May I help on filament life issues? I am not too familiar with the CM Series but I would have thought they provide an emission current (beam current) meter as do other manufacturers. However I do not know any manufacturer in the EM business who would even guess at the filament temperature under the wide range of operating conditions offered. What puzzles me with your question is the heat applied to the filament is just part of the gun saturation process, filament current, bias setting (emission current required) and filament position being the three factors which relate to the saturation point. I have analyzed filament problems on EMs for over 40 years and have never had cause to relate to actual filament temperature so may I offer some ideas? 1. As the filament ages it becomes thinner and will eventually break at the side which is slightly thinned during the bend. The normal break in a TEM should be through a gentle taper being shown on the failure area. 2. A poor vacuum in the gun will cause high levels of evaporation and contamination and the shortening of the filament life. Do not take notice of the vacuum gauge reading as this is almost certainly well away from the gun area. 3. From time to time all manufacturers suffer from being supplied with poor quality wire made up of metal and a considerable amount of rubbish. Short life, a smelly chamber and a very dirty cathode assembly are indications of this problem. 4. Finally we have situations where the operator just does not know what they are doing when saturating the gun and they overheat the filament. I wrote an article for *Microscopy Today* some years ago that related to "The Life and Death of the Tungsten Hairpin Filament" which may add information to your fault finding procedures? **Steve Chapman protrain@emcourses.com** Wed May 26

Thanks for your reply Steve. I'm using LaB₆ and CeB₆ filaments, not Tungsten. They prematurely failure at around 700 hours, when the manufacturer says they should be lasting 2000+ hours. The manufacturer has been helpful so far, and I just wanted to answer their questions about operating environment. They may not be that familiar with TEM applications. We increase the bias setting as the filament dims over its life, usually requiring an increase in

heating current to maintain saturation- I think this is why most fail eventually from the insulators melting. But I don't know how else to maintain the required beam strength when the filament becomes dimmer. The emission current is always fairly low—again I only have the scale the microscope has, but it is around the 5% mark for most of its life, then this may increase to a maximum of 10% when it has got really dim, and we are on a high bias setting (e.g. 4 out of 6—see how useful these numbers are to a non-Philips user!). We are using 500 μm wehnelt apertures, and set the filament tip 150 μm below the outer surface of the wehnelt aperture. I have tried using a greater distance to the Wehnelt, but the beam was not strong enough for our uses. We recently tried CeB₆, but they had about the same life as LaB₆. I don't think we have unusual requirements for beam intensity, so I am puzzled as to why the filaments are not lasting as long as the manufacture claims. **Ben Micklem ben.micklem@pharm.ox.ac.uk** Wed May 26

I have been using the LaB₆ mini-Vogel mount you show for many years on a CM30 300KV TEM. My observations are: 1. Almost all failures are operator induced. Generally failure is due to heating too rapidly. The LaB₆ chip is very brittle and subject to damage by thermo shock. Do your routine run up as you would a new filament, i.e. Slowly. 2. The LaB₆ is very sensitive to the vacuum level. Evaporation is markedly increased with a slightly degraded vacuum level. The most likely leak is SF₆ which is most easily detected by the smell when you open the gun chamber and by the amount and color of the deposits on the anode. 3. Improper conditioning may result in a hard flash over. Consider the voltage and the HV cable capacitance and you can see a large quick discharge carries a high energy density, resulting in actual filament shift or work function damage. Either way it requires mechanical realignment of the tip or most of your beam will be coming from the side facets rather than the tip. Of course there are a lot of other possibilities but these are the main culprits in our facility. To answer your direct question, we expect and get about 2 years per filament. **Fran Laabs fclaabs@iastate.edu** Wed May 26

SEM: backscatter electron intensities

*From a backscattered electron (BSE) image, can the yield or intensities be quantified to give a fairly accurate number? It will be on a binary alloy of metals. Does anyone know if that can be done? **Tommy Derflinger gtuser@comcast.net*** Sat Jun 12

I would think so but only for that particular capture. Which would mean that it is actually relative to the other elements in the image. One would need a known standard along with the specimen to make any headway towards a meaningful quantification. In any case, quantification would just mean what the pixel intensity is at any point on the image. I don't see what use this would be other than to say that element x is twice the intensity of element y. Interesting, but so what? Just as with film and a densitometer, contrast and brightness will change all elements' intensity. Of course one can obtain the same type of "quantitative" results but again, they are relative to that piece of film and/or print. What is the end purpose? I would think that EDS mapping is going to provide true quantitative results for a specimen. Having counts provided at each pixel is quantitative. **Gary Gaugler gary@gaugler.com** Sat Jun 12

You may want to check this paper: Validation of quantitative backscattered electron imaging for the measurement of mineral density distribution in human bone biopsies P. Roschger, P. Fratzl, J. Eschberger and K. Klaushofer, *Bone*, Volume 23, Issue 4, October 1998, Pages 319–326. **Vladimir M. Dusevich dusevich@umkc.edu** Mon Jun 14

SEM: materials demo

*In a turn of events that will make many of you smile knowingly, I've been asked to give a half day, hands-on "workshop" on "EM" as a professional development activity for some local high school science teachers. It's a great idea, but, as you all well know, there is just no such thing as throwing something on the SEM or TEM for a "quick pic"! Thankfully, we have an SEM with LV capabilities so that we can look at unfixed samples! Some of these teachers are physics teachers and so probably not so excited about looking at the biological samples with which I am well-acquainted and bringing the micrographs back to their students. Do any of the materials scientists out there have a suggestion for an easy and easily available materials sample to look at with these teachers? **Kristen Lennon kamlennon@yahoo.com Mon Jun 21***

Several years ago a group from Dupont in Delaware gave a presentation at the Philadelphia Society for Microscopy, mostly biological focused. They showed SEM images of failure analysis that was of interest to all in that they could not show failure analysis of their work projects. Some that I remember were a screw that had to top twisted off and a broken flusher handle from a toilet. These showed force and corrosion. Perhaps you have something broken like these at home. **Patricia Stranen Connelly connellyps@nhlbi.nih.gov Mon Jun 21**

Two low prep samples that show a nice fusion of biology and physics/engineering principles are a butterfly wing scale and the structure of a chicken eggshell. I have images on my website. Neither requires critical point drying; just mount and sputter. The eggshell is the surface exposed when the inner tough membrane layer is peeled off (bet someone knows the official name...), but both sides are interesting—outer surface shows the various fine CaCO₃ beads that scatter light so nicely... The xylem image is a confocal one, but could be prepped for SEM. It is made by finding the nice weed common here - Plantago - and nicking the petiole with a razor or fingernail and pulling apart - stripping out the vascular bundles (like celery strings - that would do also...); these wall thickenings support the xylem from collapsing due to atmospheric pressure when there is greatly lowered pressure from transpiration in the leaves (how much pressure does it take to get water to the top of a redwood?) <http://www.bio.umass.edu/microscopy/gallery.htm> **Dale Callahan dac@research.umass.edu Mon Jun 21**

According to your web site, your University has a Materials Engineering department. They probably have a tensile tester and may have a Charpy Impact tester. In any case, they have the capacity to break steel samples. So ask for two pieces of the same steel, one fractured at room temperature and one at depressed (LN) temperature. People realize that things can get brittle at low temperature. The vivid difference between microvoid coalescence (overload) and cleavage (brittle) as seen in the SEM, at relatively low magnification, is something a Physics teacher or student will find immediately captivating. And if they ask you why the steel behaves like that, just look smug and inform them that they need to attend classes to find out. **Andrew Werner werner1@slb.com Mon Jun 21**

I am not a materials person, but I did have a project looking at sediment from river bottoms. The samples are very easy to prepare—just suspend the mud in a fairly dilute suspension, let the larger particles settle, then put a drop (a few μl will do) onto a carbon/Formvar coated grid and let dry. If my experience was typical, you will see diatom skeletons, mineral fragments, crystalline and not, and other items in a TEM image. EDS (if available) will also show some surprises for some of the particles. I saw U and Au in addition to the usual suspects, Na, K, Si, Al, Ti, Ca, etc. **Bill Tivol wtivol@verizon.net Mon Jun 21**

You can use paper. Place on the stub two pieces of paper: writing paper and filter paper. You can see paper fibers and filler particles in writing paper (no particles in filter paper). You can check distribution of filler with BSE and composition with EDS (usually some salts of Ca²⁺). **Vladimir Dusevich dusevichv@umkc.edu Mon Jun 21**

When I was at PPG's Glass Technology Center, we had an open house for family members. We did a demo on the SEM that was kind of neat. We set them up for a punch line at the end of the demo. Here's what we did: We took a dead fly and heavily coated it with gold. It was a really thick layer of gold. This was done prior to the demonstration. Our real sample was a cross section of enamel on glass that we wanted to show the layers and components and measure the different phases with the XEDS to show how we measure composition. To introduce them to the SEM, we showed them the fly. Scanned it all over and explained how the image was formed. We did not tell them it was coated. Of course, they thought that it was neat. Then we moved to the cross section sample and showed how we measured the composition of the different phases with the X-ray system. Oh-hum, kind of boring. Then we asked them "what was the composition of the fly?" Some would say C,H,O or whatever their guesses were. We then said, "Well, let's find out for sure." And, of course, the only thing that came up was gold in the spectrum. We then asked, "Why is it gold?" and then let them guess. At the end, if they didn't know, we asked them if we could interest them in stock certificates in the "PPG Lost Gold Bug Mine" which were certificates that we made up with a big yellow faux colored SEM image of the fly, the X-ray spectrum in the background, and the signed signatures of our group members as officers in the "PPG Lost Gold Bug Mine Corporation". I think that it went over fairly well and the kids were carrying around their stock certificates all day. **Scott Walck swalck@southbaytech.com Tue Jun 22**

SEM: air sensitive sample

*I am challenged with the task of performing SEM on air sensitive catalysts. Can anyone share their method for doing this? I have seen SEM images of the catalysts that I am interested in analyzing. However the company that generated those micrographs can not share their procedure with me. I can provide more information regarding the catalyst off-line. **Jackie Ayotte jacqueline.ayotte@ticona.com***

Somewhere I saw people using air-tight box with a cover that was kept in place by suction (atmospheric pressure), so that specimen in the box was kept under vacuum. Small spring, attached to the cover, was used to move it away when air pressure in a SEM specimen chamber was low enough. Specimen was conductive and the box was made from metal. **Vladimir Dusevich dusevichv@umkc.edu Mon Jun 21**

I have recommended the following procedure to people using our SampleSaver™ storage containers. These containers are compatible with glove boxes, so if you are using a glove box, you simply close your sample up in the container without the need to purge the unit. The SampleSaver™ container is used to transport the sample from wherever you are processing/preparing the sample under shielded conditions to the microscope. There are glove bags with large open ends that you can place the unopened SampleSaver™ container in and then tie the glove bag over the open end of the SEM. You then can fill the glove bag with nitrogen and then take the sample out of the SampleSaver™ container in the glove bag, place the sample in the SEM stage, close the stage and pump it out. I would, of course, bring the SEM chamber up to atmosphere with nitrogen and purge it while the glove bag is on prior to opening the SampleSaver™. **Scott Walck swalck@southbaytech.com Mon Jun 21**

Introducing the MICA-1600 Microcal EDS



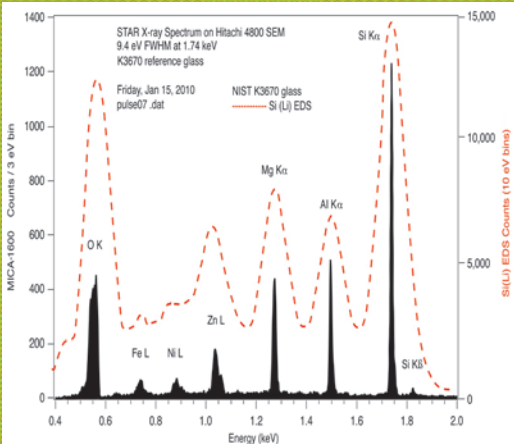
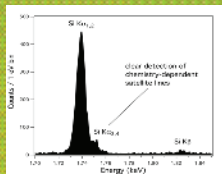
Delivers Greater Energy Resolution Better Than 10 eV and at Higher Count Rates

The new MICA-1600 X-Ray Spectrometer from STAR Cryoelectronics combines a next-generation EDS with WDS resolution into a single instrument to provide the high resolution, throughput, uptime and ease-of-use required for materials characterization and analysis. Its unique microcalorimeter easily resolves line overlaps, detects light elements and characterizes the thinnest, smallest features at very low beam voltages.

Features include:

- Energy resolution better than **10 eV** FWHM at 1.7 keV
- Count rate of approximately **10 kcps**
- A large sensing area of up to **4mm²**
- Works to within approximately **45 nm** of the sample
- Operates with a 0.1 K refrigerator that only consumes clean electricity
- Absolutely no liquid cryogens are used

For more information, contact Hideo Naito, Marketing Director, STAR Cryoelectronics today at 408-722-8478 or microcal@starcryo.com



THE MICA-1600 PROVIDES SIMPLE, ACCURATE QUANTITATIVE ANALYSIS ACROSS THE ENTIRE ENERGY BAND. SEE CLEAR DETECTION OF CHEMISTRY-DEPENDENT SATELLITE LINES.