NetNotes

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Specimen Preparation:
TEM of rat brain

I have this "weird" problem and need your help. A student here has been trying do some TEM work with rat brain tissue. She first tried the "standard" protocol for embedding: - Perfusion with 2% paraformaldehyde, plus 2% glutaraldehyde - Further fixation with 2% paraformaldehyde, plus 2% glutaraldehyde, room temperature, 1 hour - 2nd fixation with 1% osmium, room temperature, 1 hour - Dehydration 15 min each step, then extended to 30 min each step. - Transition with propylene oxide - Infiltration with 2:1, 1:2 (propylene oxide:resin), then 100% resin - Polymerization (65°C overnight) The problem is that I always saw an incomplete infiltration (at least I thought so) - We were unable to cut even 2 µm section. The sections fall apart, once it reaches the tissue. As I checked the semi-thin section, I saw holes all over the tissue. The student then tried to 1) extend the dehydration time (up to 3 min each step), and infiltration time (overnight each step) 2) additional microwave for infiltration; 3) smaller tissue (200 µm Vibratome section, then 2 × 2 mm cubes); 4) different resin (Spurr's, Embed812 and even LR white) What is amazing to me is that nothing has worked so far. Any help? ***Zhaojie Zhang zzhang@uwyo.edu Tue Feb 9***

Was the perfusion successful? The tissue should be (mostly) clear of blood and have a yellow tint due to glutaraldehyde action. How big were the pieces of tissue for subsequent processing? Very important! Low long was the dehydration in ethanol? How long was the infiltration with 100% resin? How many changes? You certainly should not be having problems after the changes you made. The problem is with initial fixation, or lack thereof. Everything hinges on good fixation. Make fresh fix with all fresh components. Do it with the student present so possible errors can be found. Does the tissue blacken with osmium? 3 min. dehydration is just sufficient, even with 200 micron sections, so that could be one of the areas of concern. Use fresh ethanol. It does not matter how long poorly fixed tissue is in resin or what resin is used. Geoff McAuliffe mcauliff@umdnj.edu Tue Feb 9

How can fixation influence infiltration? I suspect ethanol. Is it really 100%? Was it stored for more or less long time in opened bottle? Also, how many changes of pure resin? Vladimir Dusevich dusevichv@umkc.edu Tue Feb 9

That was a good question. I never thought that fixation would affect infiltration, yet several people suggested so. Does anyone know why? As for ethanol, we always use a freshly opened bottle for 100% with 3 changes. For pure resin, we make at least 2 changes (total 12 hours infiltration in 100% resin) Zhaojie Zhang zzhang@uwyo.edu Tue Feb 9

Make it ×3. Perhaps something Geoff has observed empirically? . . . If I observed such thing, the explanation I would offer would be: One of the things an aldehyde fixative does is that it makes the membranes permeable. This could be critical for efficient dehydration and infiltration. Now that I think about it—that is indeed a more widely known reason to why we fix in aldehyde to do Tokuyasu immunoEM: to facilitate infusion with sucrose before freezing. Very likely the same thing with resin embedding. Otherwise, the pieces described are definitely thin enough, and dehydration times are more than adequate, especially with propylene oxide. Interesting. Vlad Speransky vladislav_speransky@nih.gov Tue Feb 9

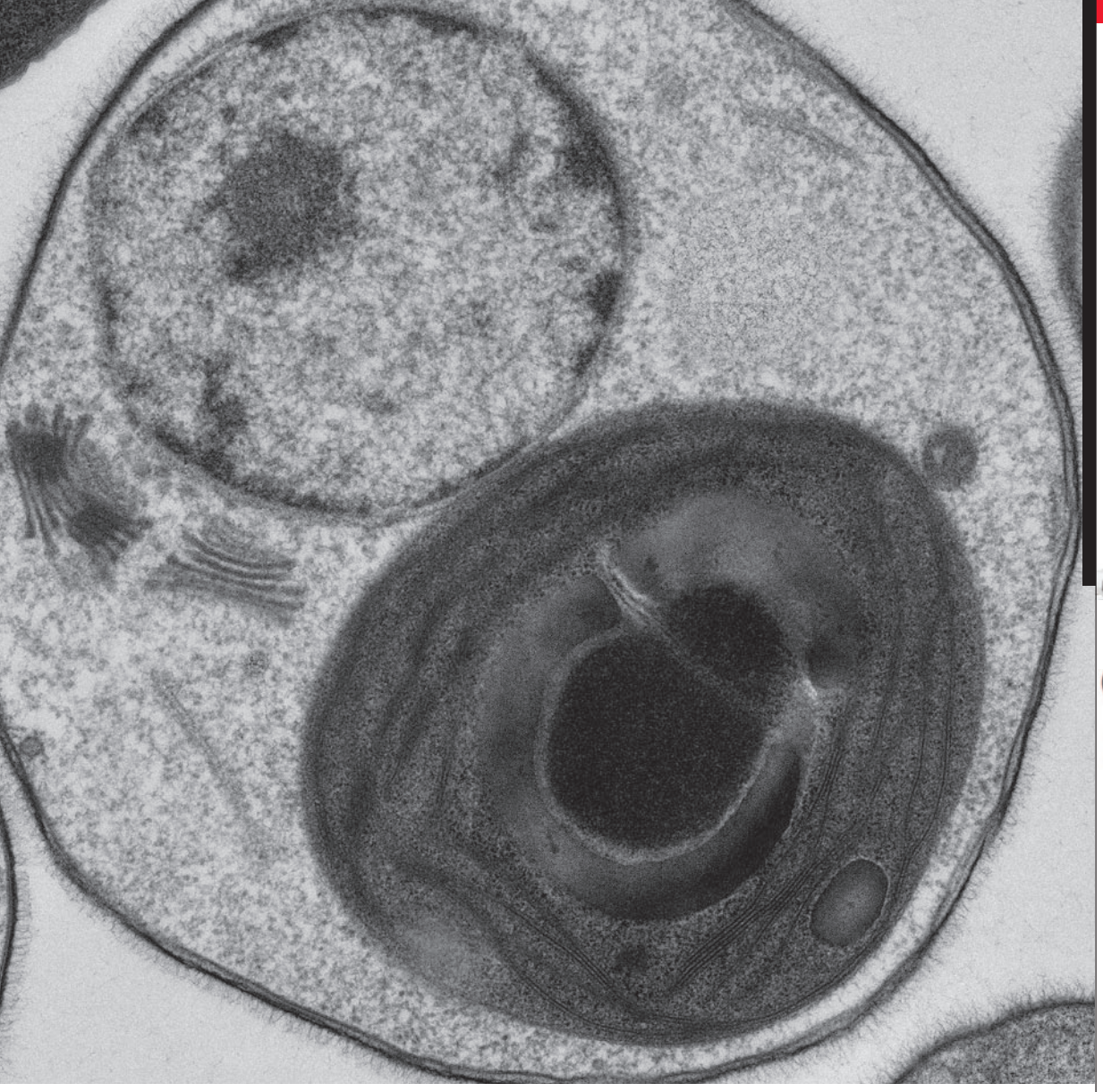
If the fixation is inadequate, proteins, etc. aren't crosslinked and locked into place, and then they can wash out during subsequent steps. Lee Cohen-Gould lcgould@med.cornell.edu Tue Feb 9

Then the washed-out area would be filled with resin, causing a structure problem, but not infiltration problem, right? Zhaojie Zhang zzhang@uwyo.edu Tue Feb 9

I can assure you that fixation has absolutely no effect on dehydration and embedding. There is no theoretical reason and that is confirmed in practice. I have been asked to embed very badly conserved material (one was a piece of rat brain!) and I was able to process the tissue as usual and to make 80–100 nm thin sections without problems. Since, in theory, your protocol looks OK, I would rather troubleshoot every single possible practical problem, especially those which are not expected to happen. This really looks like a dehydration problem. 1) Make sure than your resin is OK: ultracut empty blocks, without tissue (or embed something dry and soft, like a tiny piece of cloth). You can also simply try to cut the other end of already prepared blocks. If your resin is OK, then it is a problem of dehydration. 2) Did the student work alone? Perhaps you should assist her and see if she's doing everything all right. Perhaps during dehydration she leaves too much liquid and take it over to the next step. 3) Ethanol contaminated? Try dehydration in acetone! This is more "extractive" than ethanol, but it also dehydrates very well and that's the current problem you have to solve. You can do acetone/Epon mixes, no need for a propylene oxide step. 4) Troubleshoot the ultramicrotome: can you cut thin sections from a previously embedded material? 5) Pure resin incubation: I usually do first 3 hours, then overnight, then another 3 hours and then embedding. 2 changes for a total of 12 hours is perhaps a bit short. 6) Extend the polymerization time: at least 2 days. In practice I just start on Friday and I have nice blocks on Monday. Stephane Nizets nizets2@yahoo.com Thu Feb 11

Specimen Preparaton:
epoxy bubbles

A colleague of mine inquired about epoxy encapsulating a MEMS device for cross sectioning. The device has interlaced fingers approximately 20 µm tall attached by cantilever springs. The fingers are suspended in air above the device substrate. She has been attempting to epoxy encapsulate the part for lapping but has been plagued with bubbles being trapped between the fingers. Can anyone suggest how to accomplish epoxy encapsulation while minimizing the air bubbles? ***Guy Jackson g-jackson@ti.com Sat Feb 13***



Antarctic Algae. HPF freeze-substitutes in 2% Oso4 in acetone. Sample provided courtesy of Dr. Kirk Czymmek and Shannon Modla, Delaware Biotechnology Institute Bio-Imaging Center

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Is she using vacuum to remove most of the air? We have a device from Struers for that purpose. It should reduce the size of the bubbles if not eliminate them. There are also devices to apply pressure to the epoxy after pouring. That could be used after vacuum impregnation and would serve to reduce the size of the remaining bubbles even further. I am not surprised that bubbles are trapped without these techniques. However, sometimes, a suitably non-viscous epoxy can be poured slowly enough to allow the air to move out of the way. Some epoxy formulations are quite viscous and it may not be possible to pour them slow enough to get rid of the bubbles. Warren Straszheim wesaia@iastate.edu Sat Feb 13

If the materials are compatible with a solvent like acetone (etc.) you can use a dilution of the epoxy with solvent to aid wetting and penetration and then let the solvent evaporate in a fume hood draft to thicken the resin. We use this method for infiltrating fixed biological samples but it should help in this case as well. After the resin has reached ~100%, remove as much of the resin as possible and replace with fresh resin and allow some time (preferably on a rotator—~10 rpm—to mix. A vacuum can be applied at the end as others have described. If you have a choice, use one of the low viscosity resins like the Ellis modification of the original Spurr's formulation, and even the low viscosity variant using Quetol 651, if needed. Dale Callaham dac@research.umass.edu Sun Feb 14

You might want to place the epoxy in a vacuum chamber prior to embedding your samples. You would be surprised how much out gassing you will encounter. In my experience, the longer and higher the vacuum, at this point, the better. Be forewarned, use a container that is significantly large, the frothing epoxy has been known to overflow the brim! By pre-evacuating the resin you will also find that your samples are less likely to be disturbed by the movement of air bubbles once they are embedded and re-introduced to the vacuum. I would also consider brief sonication but this avenue is totally sample dependent and might not be well suited to your applications? John Robson john.robson@boehringer-ingelheim.com Tue Feb 16

I always disliked pulling a vacuum on epoxy resin, and many, many years ago I discovered I could minimize bubbles in epoxy by first holding the molds and labels in the 60°C oven overnight, or at least for a few hours before using. I rarely get bubbles in anything that has gone through my usual fixation (for biological samples). For materials (I currently have paint chips in resin in the oven), I find that pre-warming the samples also helps and, if this doesn't do it, pulling a vacuum on them before embedding. I passed along this piece of wisdom to the mechanical engineering guys who were getting bubbles in their epoxies containing nanoparticles (another bane of my ultramicrotomy existence), and once they started pre-drying their molds, they quit getting bubbles. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Tue Feb 16

Specimen Preparation:
cultured cells

I would like to ask for advice on preparing mammalian culture cells for thin-section TEM that have been grown in suspension. We plan to use standard chemical fixation methods but how to handle the cells--can they be treated like bacteria and pelleted into agarose following primary fixation, or are there other approaches that have been used successfully? We would like to avoid repeated pelleting during processing. ***David Lowry dlowry@asu.edu Thu Mar 11***

Low melting point agarose is good. One can suspend the cells in low melting point agarose, spin them, and then put the tube on ice to harden. Another option is to soft fix the cells, re-suspend them in 0.5 ml of 0.1M cacodylate + 8% BSA and pellet. Gently add 4 drops of 25% glutaraldehyde but do not disturb the pellet and let the glutaraldehyde hard fix your cells and cross- link the BSA for about 45 min. Then, cut off the cell containing pellet and cut into small bits. No more spinning needed. I have used both methods. Each method has advantages and disadvantages. In each case, you can control the speed/length of the final spin to either maximize cell density or cell shape. David Elliot elliott@arizona.edu Thu Mar 11

Interesting approach, I take good note. However, I usually just pellet the cells once in a conical tube and then fix them and the pellet is hard enough to be treated as it is. The specimen never moves from the tubes until curing of the resin and I add the solutions gently against the tube walls to avoid dislocation of the pellet. After osmium fixation the pellets are pretty hard. Stephane Nizets nizets2@yahoo.com Fri Mar 12

Specimen Preparation:
ethanol

So, I was planning a party and went to the solvent room to get the makings. (Not really!) When I got there all I found were jugs labeled Ethanol, denatured, CDA 19. I had been wondering about this, the alcohol in the lab had a strange odor and sometimes did not seem to be the same as the 95% at other jobs. I found out that CDA 19 stands for Completely Denatured Alcohol Formula 19. Formula 19 has things like methyl isobutyl ketone, kerosene, or aviation gas mixed in so it is undrinkable. Well you can drink it, you will get sick. This is my first exposure to CDA 19 and I was wondering if anyone else has run across this and if you have any thoughts on using CDA 19 in place of traditional 95% ethanol for tissue processing. ***Jonathan Krupp jkrupp@deltacollege.edu Mon Mar 15***

I have not seen bottles labeled with CDA 19 but I will look in the Pathology Lab to see if they have it. Many years ago I learned to make all dilutions for ethanol dehydrations for TEM from 100% (99.9%) not like they do in the histology lab (LM) which used the 95% gallon jugs or bigger. I use older opened bottles for the lower percentages to save money and this works very well. The difference was quite noticeable. Pat Stranen Connelly connellyps@nhlbi.nih.gov Mon Mar 15

This doesn't address the tissue processing question but there was recently an interesting story in Slate online magazine of the government's intentional "denaturation" of alcohol during Prohibition and how it poisoned (and killed) hundreds/thousands of citizens. Check out this link: http://www.slate.com/id/2245188 The Chemist's War The little-told story of how the U.S. government poisoned alcohol during Prohibition with deadly consequences. Tom Phillips phillipst@missouri.edu Mon Mar 15

I can't speak for this specific designation but the chemical properties of the denaturant are a factor in some other fields as well. In art conservation (and to some extent in lacquer formulation) it is preferable for us to use a grade that has only other alcohols as denaturants (i.e., without ketones, petroleum distillates, etc.) One type that is readily available in hardware stores uses methanol and isopropanol as the denaturants and works better for solvating resins whose solubility is impaired by non-alcohols. I'm sure that laboratory grades of the same thing are available. You might also consider the suppliers that serve the microelectronics industry, as they have very stringent standards and are a bit off the radar in terms of academic and bio labs. John Twilley jtwilley@sprynet.com Mon Mar 15

Specimen Preparation:
TiB_2 particles

We are producers of aluminum grain refiner alloy in central India. During establishment of process parameters, we required detailed analysis of grain refiner (TiBAl) microstructure. TiBAl aluminum alloy contains Ti and B as main additives. Typical grain size is 30–50

micron for $TiAl_3$ and 1–2 micron for TiB_2 particles. Kindly give us some technical feedback on developing such typical micrographs. The alloy contains Ti-5% B-1% and rest Al, the major phases produced are $TiAl_3$ and TiB_2. We draw TiBAl grain refiner in wire form. We want to evaluate the procedure for TiBAl grain refiner–fine polishing techniques. Presently we are using Buehler Alumina solution, followed by diamond paste of 3 micron. We are not able to reveal the structure properly. Kindly help us in elaborating the technique for getting good microstructures. $TiAl_3$ and TiB_2 are the two phases under analysis. How should we polish so that it will reveal the lowest particle of 1–2 micron? Will excessive polishing will rip of the TiB_2 from Al matrix? We need to evaluate the grain size and Ti/B particle distribution in the micrograph? How could we count the 1–2 micron particle with light microscope? The samples are in the form of wire with thickness of 13 mm or 9 mm. We also need to evaluate the samples under SEM EDS. Does it need still special polishing? We also need to study the TiB_2 particles under it. Earlier, we tried to study the TiB_2 particle under SEM but we faced the sample charged difficulty (i.e., we could not able to any particle structure, all screen was bright). Please provide some remedy for the same, so that we can analyze the sample under SEM easily. ***Amit Kamble amit@minexindia.com Fri Mar 26***

Buehler Co. itself is a good resource. If they contact Beuhler, they will probably get the help/guidance that they need. After polishing, some kind of etchant may help reveal particles for SEM. Ron L'Herault lherault@bu.edu Fri Mar 26

I made this kind of metallography some 30+ years ago so it may be a little out of date. Our material was made of Al 99,99% Ti was added as F_6TiK_2, B was added as F_4BH individually or together. Another form to include this elements was under the form of a commercial product "FOSECO NUCLEANT II". These materials were added to Al at 800°C. The analyzed material was as cast. Three techniques were used: 1) The free surface of the ingot was lightly polished electrolytically in a cell to clean it, and attacked with a Disa Electropol apparatus. 2) The free surface of the ingot suffered only an anodic oxidation. 3) Cuts of the ingots were mechanically polished, then electrolytically in a cell and structure revealed by anodic oxidation. Observation on the microscope gives the best results with phase contrast, Nomarski system. Also dark field illumination gives good results. Of course clear field is OK. Mechanical polish: after grinding to emery paper 600, finally polished with 7 microns diamond paste. This last step gives later a better result but can be left out. Electrolytic polishing in cell was made with Al cathode temperature kept under 10°C, time from 5 to 10 minutes cell voltage, open circuit, around 28 volts (depends of the power supply characteristics), composition of the electrolyte was: Percloric acid 10% Glicerine 3% Butilcellosolve 87% all by volume. DISA Electropol A2 electrolyte Percloric acid 78 ml. (added last) distilled water 120 ml. Ethanol 700 ml. Butilcellosolve 100 ml. When used to polish current is about 1 A/cm^2, for attack 60 mA/cm^2 temperature should be kept under 25°C Anodic oxidation was made in a cell, cathode was stainless steel (18/8), Voltage about 10 to 18 V. time about 1 minute. Electrolyte is 10% sulfuric acid in water. This oxide can be scratched with a sharp point then put into a 5% solution of mercury chloride in water. The oxide layer with the Al_3Ti particles attached will be separated and put into a TEM for diffraction or EDS. Our particles were too big for diffraction and had no EDS. The "normal" specimens were analyzed in a CAMECA WDS with good results as for Al_3TI but not for Boron. Francisco José Kiss kiss@demet.ufrgs.br Fri Mar 26

Specimen Preparation:

bacteria on a leaf

I am trying to image bacteria resting on a leaf (not in a biofilm), but when I dye with fluorescent stain/fix for SEM imagery, the bacteria fall off into the liquid of the stain/fixative and I am not able to capture them resting on the leaf. I was curious how to best prepare a sample such as this for fluorescence or electron microscopy that perhaps wouldn't involve liquid. ***Sarah Lego slego@agraquest.com Mon Mar 15***

I would suggest trying a fixation in OsO_4 vapor. There is an old method for preserving arial fungal structures published by King and Brown (1983). We are using it for samples of arial Streptomycetes mycelia for SEM. It also works well on fungal mycelium grown on conifera leafs. Here is full citation: King E.J., Brown M.F., 1983. A technique for preserving aerial fungal structures for scanning electron microscopy. Can J Microbiol 29: 653–658. Oldrich Benada benada@biomed.cas.cz Tue Mar 16

You can try to minimize specimen preparation procedure: just air dry leaves and sputter coat them. You do not need stain for SEM and quite often air borne bacteria are visible without fixation. Vladimir Dusevich dusevichv@umkc.edu Tue Mar 16

LM:

lubrication

I have inherited two compound microscopes with similar problems. The focusing oculars of one are very tight so the rotating mechanism needs to be cleaned and re-lubricated. In the other scope, it is the condenser field diaphragm that is tight. Does anyone have ideas on how to approach this task in regards to cleaning and what lubrication to use? ***Tom Phillips PhillipsT@missouri.edu Thu Feb 25***

Take it apart (maybe make drawing or marks for later assembly) sonicate parts in solvent if possible. Lubricate with light grease or oil sparingly, avoid getting it on optical parts. It maybe is tricky, but doable to take the focusing gears apart or at least partially. Use penetrating fluid like WD-40 and/or sonicate, use a pipette for WD-40, re-grease and assemble. Markus F. Meyenhofer micro@superlink.net Thu Feb 25

Not too much help, just "dismantle carefully", and I have used lithium grease to lube microscope parts in the past. Cleaning is Q-tips & ethanol and the like. And wiping Philip Oshel oshel1pe@cmich.edu Thu Feb 25

Disassemble, clean all metal parts with toluene to remove old grease. Swabs, hood, gloves, etc. Some Nikons from the 70's have greased plastic parts. Alcohol is o.k. on these, but it's slow to dissolve grease. Go gently on re-greasing plastic. It is usually there because it didn't need grease in the first place, and some aftermarket microscope tech put some on anyway, trying to be helpful. My favorite general microscope lubricant is an ancient 2 oz pot of Nyogel 764C (Nye Lubricants, Bedford Mass). I don't think any whales were injured in its production, but it's almost that old (early 60's?) The fact that it's still good, non-hardened, and non-oxidized should give you an idea of how long the stuff lasts in place. No lubrication on iris diaphragm leaves ever. If you're really careful, a small dot on the leaf pin is o.k. But if there's any chance it will migrate out onto the leaf itself (high heat, such as a fluorescence illuminator), none at all is better. Julian Smith smithj@winthrop.edu Thu Feb 25

I normally clean out the old oxidized grease with a 1:1 mix of ethanol and ether. Then reapply lubricant "Nyogel" medium damping grease from Nye Lubricants Inc. Russ Spear rzs@plantpath.wisc.edu Thu Feb 25

This problem is usually caused by polymerization or drying out of the lubricant, resulting in metal upon metal rubbing of the moving parts. You need to disassemble the part, taking care to remove, if possible, the optics safely away from the metal parts. Try then soaking the metal parts in xylene to loosen or dissolve the grease. Then, clean the parts with cotton swabs or lint-free cloth soaked in xylene. You

may have to repeat this soaking and rubbing many times to get through the built up grease. When sufficiently clean, the parts should move smoothly against each other, even without lube. Finally, apply a very thin layer of lithium grease on the moving parts. I've cleaned many microscopes over the years and failed to get only a couple parts working again (a microscope focusing stage and a condenser) since I was unable to disassemble them. John Bozzola bozzola@siu.edu Thu Feb 25

The perfluorinated polyphenyl ether oils (Fomblin and Krytox) and greases (Brayco and Krytox) are superb lubricants, and they are chemically inert and do not oxidize or polymerize. They also have very low vapor pressures (10^{-10} Torr or better) and can therefore be used as lubricants for things inside a vacuum system. As I recall, the guy that services microscopes here at the U of Mich. has been using these very successfully for a number of years. Wilbur C. Bigelow bigelow@umich.edu Thu Feb 25

Image Processing:
image colorization

I'm looking for a Free Software program that can take SEM/FIB TIF images and colorize them at designated areas. Any ideas? I've tried the usual free image programs but so far I've found none that can easily do it. ***Ian Drucker ian.drucker@gmail.com Wed Feb 24***

There is no easy way to get precisely colored images. Best would be to use various detector-signals grabbed at the same time to get rid of pixel-offset and blend them over via layers and / or paths in Photoshop or any other software able to handle image data. Stefan Diller stefan.diller@t-online.de Wed Feb 24

I don't know how much of automation you need, but The Gimp (http://www.gimp.org/) is doing quite a good job in image manipulation and its open source. Guenter Resch guenter.resch@imp.ac.at Wed Feb 24

Stefan is right, and it really is pretty easy, if time consuming, to do in Photoshop CS4. On a really tight budget the likes Serif PhotoPlus X3 or Adobe Elements 8 will suffice. There's always different ways to approach something like this in Photoshop CS4 [or earlier versions], but I'd try: Load the SEM Photo and manually select the region you wish to colorize, using say the 'quick selection tool'—you can add/reject [shift/alt] bits of selected regions with a lasso tool. Then ensure the image is RGB color [image, mode]. I'd then go to 'image, adjustments, color balance' and lightly adjust the color balance of the selected region and then "you could be brown, you could be blue, you could be violet sky", e.g. move the color balance slider from cyan to red and the selected object with become progressively more red, with the underlying structural details intact. Then work through the entire image. Select all objects of one color as one multiple region for colorizing [shift], and you can say slightly adjust contrast and brightness, or curves, or shadow/highlights within those selected regions as well. You don't really need layers, you could work on the main image [background] bit by bit—save regularly under new file names as Photoshop's undo [step backwards] function is limited if, after a lot of work, you don't like the way the image is turning out. You could do all this with Photoshop Elements 8 as well. Do all the above Photoshop stuff using the similar Elements tools, then [instead of 'color balance'] go to: Enhance, Adjust Color, Adjust Hue/Saturation [and make sure the 'Colorize' box is ticked]. It will take a while to manually edit the entire image ['View, Zoom' to aid tracing], but I doubt any image processing software could fully 'automatically' select the regions you want to colorize, particularly with a '3D' SEM image. Photoshop's 'Quick selection tool' will have a go though [the tools selection effect is adjustable in the upper menu bar]. I doubt the likes of 'Pseudocolor' LUT effects will provide the subtle coloring you require, although they may work adequately on TEM images of sections. Keith J Morris kjmorris@well.ox.ac.uk Thu Feb 25

There aren't any free programs that I am aware of for pseudo colorization. The paid programs will do the whole image but not selected areas. If you use Photoshop, you can colorize selected areas and as different colors. Gary Gaugler gary@gaugler.com Thu Feb 25

Tried out colorizing an SEM image with Photoshop CS4 and the results seemed fine: Original SEM image of pollen http://en.wikipedia.org/wiki/File:Misc_pollen.jpg. I colorized the image using the method in my last post. You could use Photoshop CS4 or Elements 8. See http://www.well.ox.ac.uk/cytogenetics/pollen/pollen_colorized.jpg. It took well under an hour to select and colorize the whole image and I found it quite therapeutic. I also applied a Pseudocolor look-up table [LUT] applied using Photoshop CS4 Elements 8 has similar tools. http://www.well.ox.ac.uk/cytogenetics/pollen/pollen_pseudo.jpg. See details of using pseudocolor LUTs with Photoshop and the likes of http://www.rawlight.com/psuedo.pdf. Search the internet; I have seen one free image editor that apparently can colorize images fairly well. It's used by artists, and some of the results are pretty stunning, if a little gaudy. But the editor interface seems very clunky and I'm not sure how to upload and save the images using it. It talks of your edited images being available for all, i.e., you might lose copyright or at least your images might be free for others to view or steal. http://aviary.com http://www.well.ox.ac.uk/cytogenetics/pollen/pollen_dayglo.jpg http://fxh.worth1000.com/entries/507038 http://lifehacker.com/5309162/best-online-image-editor-aviary-phoenix. But it is apparently free to use. However any opportunity to hone your Photoshop skills is probably worth taking. Keith J. Morris kjmorris@well.ox.ac.uk Fri Feb 26

In addition to GIMP, there is a modification called GIMPshop that attempts to make GIMP look and feel more like Photoshop. http://en.wikipedia.org/wiki/GIMPshop Most notably, GIMPshop allows the use of Photoshop plugins, which can be useful. Marc Takeno takenomm@u.washington.edu Tue Mar 2

Corel Paint Shop Pro Photo is also a very powerful program that will do (just about?) everything Photoshop will do for about $100 US. I even got a 2 GB USB drive with my copy. Geoff McAuliffe mcauliff@umdnj.edu Wed Mar 3

Image Processing:
digital tablet for particle size

I am interested in using a digital tablet to collect particle size data (average particle size, aspect ratio, etc) from TEM images directly on digital image or on printed image if necessary for tablet. The particles are sub micron in size and tend to agglomerate which makes image analyses extremely challenging (many overlapping particles). I feel that if I could outline the particles using a digital pen / digital tablet I could then collect some semi-quantitative data. I'm hoping someone out there has used this technique and can give me some guidance. Direct advice or suggested publication of techniques would be greatly appreciated. Any other suggestions for collecting particle size data from TEM images would also be welcome. I am familiar with Image Pro Plus and use it routinely for non-agglomerated particles. ***Sandra Gardner sandra.gardner@xerox.com Thu Feb 18***

What you are suggesting is quite simple. The only problem is that what is easy for the human is difficult for a computer. I use Wacom tablets for 'manual data segmentation'. An easy (not the best, but very easy to learn—I use high school students or undergrads to do the work) way to do this is to open your image in Photoshop (or some such program). Using your digital pen or mouse, select the pencil tool and set the tool size to something easy to use, but smaller than your particle. Create a new layer. Draw the particles you want to analyze

in the new layer. Save this document. This is the record of what will be analyzed. Create a new document with only the drawing layer. Open this in NIH image (ImageJ) and "Analyze Particles" You can get all kinds of information out of this analysis. If you want more info, please contact me. David Elliot elliott@arizona.edu Thu Feb 18

This can be done quite easily using just ImageJ, eliminating the need for a second program and the extra steps. 1. With image in ImageJ (I assume you have calibrated it) outline a particle, and press 't' to add it to the ROI Manager. 2. Continue outlining and pressing 't' for each particle. Be sure "show all" is checked so you can see what you've already selected. 3. In the ROI Manager window, the 'More' button will show a choice to save the selection set if you need it for record-keeping. Not necessary for analysis. 4. Also in the ROI Manager, you can select "Multi Measure" to measure each selected area Note that I didn't get into image calibration or selection of which measurements you want to perform on the particles. I just wanted to show it was easily done. I'm sure Image Pro will also have the capability, but I rarely use it so I can't give instructions. Jim Passmore james.passmore@sealedair.com Fri Feb 19

Use a Sharpie pen on clear acetate sheet (remember the old overhead projector sheets?) to trace the outline of your particles. Then digitally scan the tracing. Not any slower than using a digital tablet to trace, and I like it because I can move the acetate sheet after tracing one particle and then trace the overlying particle fully onto a blank area of the sheet--no overlapping particles on the tracing. I am learning to write left-handed now because I have worn out my right hand doing so many grain boundary tracings. Roger A. Ristau raristau@ims.uconn.edu Fri Feb 19

Editor's note: See R Anderson, "A Very Simple Method for Quickly Making Large Numbers of Measurements on Micrographs," *Microscopy Today* **17**(3) (2009) 50.

EM:
textbooks

Does anyone have any suggestion/recommendations on good textbooks on Biological SEM and TEM? I'm looking for these and there are many out there and I am not sure which one to get. ***Fiona Chia fionacxr@tll.org.sg Fri Mar 19***

I may not be aware of all the books out there now but if I could have only one textbook to cover the essentials of the microscope hardware and vacuum systems and clear, well illustrated, details of specimen preparation, imaging, interpretation, I would definitely get the Bozzola and Russell book "Electron Microscopy, Principles and Techniques for Biologists". It clearly covers the basics and much more. A few copies are sometimes found on Amazon and eBay. There is an older edition that still covers the basics very well. Our university has an electronic version subscription which is another way to get it. Dale Callaham dac@research.umass.edu Fri Mar 19

I second the suggestion of Bozzola and Russell. Here are a few others that I like. Hunter "Practical Electron Microscopy"—mostly beginner stuff, but great for beginners. Maunsbach & Afzelius "Biomedical Electron Microscopy"—this is the first book I look to for answers and suggestions. Crang & Klomparens "Artifacts in Biological Electron Microscopy"—self-explanatory. David Elliot elliott@arizona.edu Tue Mar 23

I like "A beginner's handbook in biological transmission electron microscopy", 2nd edition, by Brenda S. Weakley. Geoff McAuliffe mcauliff@umdnj.edu Wed Mar 24

There are a lot of really good texts by Springer Pub. As for SEM I recommend the following book: Scanning Electron Microscopy and X-ray Microanalysis. Joseph Goldstein, Dale E. Newbury, David C. Joy, Charles E. Lyman, Patrick Echlin, Eric Lifshin, Linda Sawyer, J.R. Michael. Springer, 2003. For TEM, I recommend: Introduction to Conventional Transmission Electron Microscopy. Cambridge University Press, 2003. Another TEM textbook for materials engineering: Transmission Electron Microscopy: A Textbook for Materials Science. David B. Williams, C. Barry Carter. Springer, 2009. For TEM sample preparation: Handbook of Sample Preparation for Scanning Electron Microscopy and X-Ray Microanalysis. Patrick Echlin. Springer. Ahmad Ashkaibi ahmad_ds@yahoo.com Fri Mar 26

EM:
location of lab

My workplace is moving into new buildings, which are not yet build. I mentioned to some of the people involved in this planning process that the EM-lab need to be situated in the îbasementî, and then the question came up if it is possible to make the lab on the ground floor, or if the vibration in the building will be to disturbing. The labs I have visited have always been localized in the bottom of the buildings. Does anybody have any thoughts? ***Lene Hermansen lene.cecilie.hermansen@veths.no Fri Mar 26***

Realize that all structures have natural vibration frequencies. The vibration level in a basement, where the floor is most likely poured concrete on undisturbed soil, is probably less in magnitude and of a lower frequency than what would be in the building floors suspended above by the building structure. The vibration of equipment within the building only adds to these vibrations and issues. The basic goal, as I understand things, is to place the microscope on a sufficiently massive base that the fundamental vibration frequency of the base is well below the fundamental frequency of the microscope structure. Thus the base tends to dampen any vibration frequencies of potential harm to the scope. In a basement, the ideal would be to excavate a large amount of soil and pour a large block (mass) of concrete for the microscope to ultimately sit upon. This block should be mechanically isolated from the surrounding concrete floor and building structure (not solidly connected). On upper floors, this large mass is difficult to achieve for some obvious reasons of weight and size. There one resorts to vibration isolation platforms, either passive (moderately costly) or active closed-loop systems (quite costly). My personal experience is with a microscope on the main floor of a 1940's building (thin concrete slab on soil) and I have vibration issues coming from the building and plant equipment. We have installed a passive air-sprung isolation platform ($7K US) that helps to some extent but isn't a cure-all. If I were given the choice between a basement and upper floor, the basement would definitely be the desirable location. If you are in the early stage of construction and can get a separate isolation block poured, all the better. Rick Ross richard.ross@allisontransmission.com Fri Mar 26

Others have talked about vibration, which was the point of your question, but there are these days more difficult problems! If you intend to become involved with the high performance field emission SEM that are now available from all of the manufacturers, you should play particular attention to magnetic fields. Whilst there are a number of pretty good anti-vibration systems available for minor problems, there in my experience does not seem to be devices for compensating magnetic field that are anything like as good! So keep an eye on high current carrying cables, lifts, local heavy current drawing machinery etc, as well as giving due consideration to vibration. I once put a TEM on the 6th floor of a building, taking great care to be involved with the design of the building to optimize the microscope position. I noted that the 7th floor was the animal housing facility; however, not until we installed the microscope did I find that at each end of the animal house were fans 7 feet across (air vibration, floor vibration and magnetic fields). Fortunately for me, I understand they have to

this day not used the fans. Good luck Steve Chapman protrain@emcourses.com Fri Mar

Contact the microscope manufacturer and ask for their installation requirements. This should list the vibration requirements, as well as other important things like temperature stability and e-m field limits. Give this to the architects, so that it forms part of their specifications for the building. If you have high-spec microscopes, watching their reaction can be quite interesting! Something you may need to specify to meet e-m field specs, which is easy to miss—lightning conductors must not form ground loops. This means if there are multiple conductors they must be connected at the bottom, not the top (opposite to the usual way of doing things). Richard Beanland contact@integrityscientific.com Fri Mar 26

It should be noted that manufacturers have significant pressure to develop "lowest common denominator" environmental performance specifications because they don't want to lock themselves out of the competition with regard to a prospective customer's lab space that happens to be less than ideal. When we designed our underground lab space for nano-characterization in Oregon the first thing the architects asked for were the vendor's "cut sheets" for each instrument. But I explained to them the above mentioned concern and convinced them that these vendor installation specs were only a starting point for vibration, acoustics, EMI, temperature control, etc. What we tried to do was design the entire analytical section of the building for ultimate performance based on significantly exceeding the specs of the most sensitive instrument we planned on (the FEI Titan TEM). That meant designing the floor poured directly on bed rock 20 feet below the surface, oversize round ventilation ducting for low speed non-turbulent air flow, specially engineered high sensitivity temperature control systems (based on off the shelf components), electrically isolated rebar in the slabs, all steel electrical conduit and extra shielding for all electrical panels and devices, 12 volt DC lighting for instrument operation, dedicated low noise earth grounds, all building transformers, water handling, compressors, exhaust and supply fans all located in other nearby buildings and plumbed over reasonable distances to our isolated building which has a turf roof and in which we are in the basement sitting on Eugene Formation bed rock. In fairness I should point out that this Eugene Formation bedrock is serendipitously a perfect foundation material for such a purpose due to it's porousity and high damping coefficient. In fact, one vibration consultant claimed that this site, in the middle of our campus, was the second quietest site he had ever measured in his career and when we asked what was the quietest site he said: "well, there's this mountain top in New Mexico that is 160 miles from the nearest city . . . that's a little bit quieter". What we ended up with was beautiful, but in space of my career it was a one of a kind perfect storm of support from the. More technical stuff can be found here, but let me just point with some pride that our floors exceed NIST-A vibration levels by several factors: http://camcor.uoregon.edu/fac_tour.shtml The result is that all the instruments in our facility exceed their factory performance specifications in multiple ways (or if they don't, it sure isn't due to the building environment!). For myself as an EPMA guy, it is the 0.3°F temperature control that gives my Bragg spectrometers the reproducibility I've always dreamed of. How much did this 30K sq feet building cost? About half the cost of all the instruments housed in it, and well worth it I might add. John Donovan donovan@uoregon.edu Fri Mar 26

TEM:
zero-loss peak drifting

Our newly-installed JEOL 2100F is equipped with Tridiem system. The annoying thing is that zero-loss peak (ZLP) is always randomly drifting with time, for instance, a few eV in half an hour. I would like to ask you for advice how we can improve that to keep zero-loss peak more stable. ***Yu Zongsen yu_zongsen@yahoo.com Fri Mar 19***

ZLP drift can be due to many factors. It would be a good idea to track the ZLP over a long period of time and see if the drift correlates with time of day, happenings in the building, or time after starting the analyses. We have a much older GIF 200, and we have drift which we could correlate with: 1) time after switching from image mode to spectroscopy mode, 2) cooling (on a 90 minute cycle), 3) charging at the bore at the bottom of the projection chamber. We placed a dedicated chiller on the GIF, then placed an Al flange in the projection chamber bore. These helped with drift, but did not eliminate it. My best data is collected when I can place a sample in the scope the day before, set up the analysis conditions, switch on spectroscopy mode and align the GIF. In the morning, the ZLP is more stable. Not everyone has the luxury of doing this, but when the data is important, you go the extra mile. Ken Livi klivi@jhu.edu Fri Mar 19

For better stability it is important to leave the HT at 200 kV overnight and not to reduce it. The HT tank will cause drift as its temperature changes after bringing it up and this may occur for periods of up to 6 hours after reaching 200 kV. This drift has negligible effect on normal imaging, only EELS. If your microscope has a camera chamber for film the prism that puts the numbers onto film may be charging. This should be given a thicker conductive coating, or, if film is not being used, remove it. Finally I assume you do not have steel chairs near the microscope? Wheeling a steel chair in front of the GIF will cause a shift of several eV. We have a wooden chair with no wheels that is placed in front of the microscope to stop this effect! Alan W Nicholls nicholls@uic.edu Mon Mar 22

TEM:
sampling and magnification

I study nanoparticles by transmission electron microscopy. After examining several samples I have faced to two questions: 1. What is the minimum magnification which is required to notice a nanoparticle? 2. What is the minimum magnification which is required to describe the shape of a nanoparticle? The answer to the first question seems to depend on the particle size, the resolution (number of pixels) of CCD camera, and probably the physical size of CCD (according to the Nyquist theorem). Is there a formula to calculate the minimum required magnification? The second question seems trickier than the first one (at least for me). To my mind, there should be at least two formulas (one for the minimum size and the other one for the maximum size of a particle). Could you please help me with these questions? I was trying to find answers on the Internet, but I couldn't find any concise examples, neither the formulas. Thanks! ***Dmitry Bagrov dbagrov@gmail.com Fri Feb 5***

The only formula you need is the multiplication sign. Let's say that 1mm is a "comfortable" size to notice a particle. if your particle is 10 nm in size, you'll need a 100,000× mag to make it appear 1mm big on your screen. (1 mm = 1000 μm = 1,000,000 nm) Knowing that, I think you can guess yourself at what apparent size you are able to describe a shape. NB: "comfortable" is probably not the right word, because your particle will still look like a small spot on the screen. Stephane Nizets nizets2@yahoo.com Fri Feb 5

Thanks for your quick reply! Actually I was asking from a more "theoretical" point of view: the required magnification doesn't depend only on the particle size and the size of the screen. One of the reasons is that the resolution of the microscope is finite and increasing magnification doesn't always allow one to measure the size and analyze the shape of small particles. Let's say that X is the number of pixels in the CCD camera (typically 1000 or 2000), L

is the physical size of the sample which is projected on the CCD camera. If we increase the magnification, L decreases proportionally (the analyzed area becomes smaller). According to the Nyquist theorem the minimum size of particle that can be noticed is 2L/X. Let's assume that I need 5 × 5 pixels to describe the shape of a particle (I am not sure if it is correct). So if the magnification is constant (L is constant) I will be able to describe the shape of a particle which is larger than 5L/X. If a microscope provides a 300 300 nm image with 500,000 magnification and I have a 1000 × 1000 pixel camera I will be able to describe the shape of particles that are larger than 5 × 300 nm/1000 = 1.5 nm (if the resolution of the microscope allows me to do so). Is there a way to make these or similar estimations in general? How many pixels are needed to describe the shape of a particle. Dmitry Bagrov dbagrov@gmail.com Fri Feb 5

I would suggest reformulating and simplifying the question. The size of the particles is not really so important since they are quite small and you'll easily fit at least one of them in one image. Decide first at what level of detail you want to image the particles. That is the spatial resolution you need. Let's say you want 1 nm spatial resolution. That means that the pixel size mustn't be bigger than 0.5 nm (according to the sampling theorem). To be on the safe side and minimize interpolation errors (if you want to perform averaging) you should choose a pixel size of about 0.3 nm. If your detector or scanner has a pixel size of for example 25 micrometers (25,000 nm) the magnification between specimen and detector should be about 25000/0.3 = 80,000×. If you want 0.2 nm resolution, you'll need 400,000× magnification. So the formula for the correct magnification is: detector pixel size divided by (desired resolution times 0.3) (with everything given in the same units) Philip Koeck philip.koeck@ki.se Fri Feb 5

You are headed in the right direction although your steps are mildly confusing. As far as the number of pixels required to determine "shape", just consider what an extremely pixilated image would look like. Something 5 pixels long would look very blocky. You might be able to get a sense of it being either an elongated particle or round, but you won't be able to tell much more. As Philip said it, you may wish to rephrase the problem as the level of detail that you want to see on the feature. That could be texture or shape. Then, convert that dimension into pixel size. Nyquist would say you need to oversample by two-fold. Practically, you want something that you can see and only a two-fold oversampling will leave you wondering. Somewhere along the line, you will need to bring in the practical resolution of your scope. What can you really achieve compared to pixel size? Increasing the magnification will reduce the pixel dimensions, but once your pixels are on the order of the resolution, then you have crossed over into the realm of empty magnification. More pixels in the feature will not be helping you. Warren Straszheim wesaia@iastate.edu Fri Feb 5

You might do better to consider the old TEM process of shadowing. You could evaporate Pt from a low angle, and try again to resolve the particles with the SEM. However, you may also be getting very close to the resolving limit of your instrument. Do you know what the spot size is? Joel B. Sheffield joelsheffield@gmail.com Fri Feb 5

TEM:
magnetic materials

I would like to know how to prepare TEM samples of magnetic materials and how to evaluate if the TEM samples are not harmful to microscopes. Magnetic materials may be attracted by a pole piece. Therefore, they can damage the microscope. However, we know many people have characterized magnetic materials using TEM (e.g., bacterial magnetites and thin films). I also have experiences looking at maghemite particles from soil and magnetite inclusions in minerals. I was able to see the materials without any damage to the microscope. The materials could stay on the grids. It may depend on the particle size and the material how the magnetic materials are harmful to the microscope. I would like to know: 1. How to prepare ion milled sample that is not harmful to scopes. I am afraid if ion milled samples are broken inside TEM and attracted by the pole piece. 2. Can I bring powder samples (magnetite) into microscopes? 3. How to make sure that the TEM samples are not harmful to microscopes? 4. Any other technique you recommend? ***Hiromi Konishi hkonishi@wisc.edu Tue Feb 9***

1. As you say, many people study bulk magnetic specimens inside the TEM, indeed some microscopists use magnetic materials as support grids (such as Ni). As long as your sample holder is a design which rigidly holds the specimen in place (such as a hex nut, rather than a plate and/or arm clamps) the bulk of the specimen will not be pulled out of the holder! As for thin regions being torn away, there is some possibility of this occurring—but I am sure the risk is small (those who work on bulk magnetics, please correct me!). It will of course depend on the mechanical properties of the material in question, with brittle materials being more of a problem. 2. Powdered magnetic samples are not a problem, as long as they are suitably small for TEM study (<100 nm). Van der Waals forces are much more powerful on these length scales than magnetic moments and wouldn't detach from your grid. 3. I'm not sure what you mean by harmful? What you will experience with bulk magnetics is a misalignment of the instrument every time the specimen is tilted or moved a large distance. Simply as the specimen acts on the electrons in the beam and interacts with the field of the lens. I would recommend the lowest resolution instrument you can find for bulk magnetics, as the wider the polepiece gap the smaller this effect will be. And don't even think about an aberration corrected microscope! None of these misalignments will be permanent so no 'harm' will occur. If you managed to tear a specimen out of a holder (unlikely, see point 1) it might potentially lodge in a awkward place on the pole piece blocking the beam or apply an aberration to the lens. In this situation powering down and venting the pole piece would be required to remove the specimen. Any thin area/nanoparticles removed onto the polepiece will have an inconsequential effect on the microsope, their volume is simply too small. 4. If you are really concerned about operating in a field you can use a microscope with a Lorentz lens—turning off the objective and working in a field free environment at the specimen. However, with such far field focusing you will lose a large portion of your resolution. Matthew Weyland matthew.weyland@mcem.monash.edu.au Tue Feb 9

While it is conventional to operate a microscope with the specimen stage in the eucentric position it is not essential to do so! With your magnetic materials, the lower the lens strength, the lower the level of problems as have been pointed out. 1. The "higher resolution" option is to adjust the eucentric stage so that the specimen is moved away from the lower objective pole piece which would mean a lower lens current/magnetic field during operation. Simply adjust the Z prime (eucentric adjustment) so that the specimen remains in focus as you turn your focus controls anticlockwise for a lower lens strength. You will probably obtain a focal length up to 2 mm longer than normal. This drop in objective lens current will require you to re calibrate the magnification system. 2. The "low resolution" option is to run in a "low magnification" or "scan" mode, when the objective lens is either switched off or run at a very low current, in this case focus is achieved with the diffraction lens. Point 1 is also a good idea for biologists struggling for contrast as it increases the specimen to objective aperture distance, hence contrast. It is also a method for obtaining "higher resolution" low magnification images as the magnification range is likely to drop by a factor of up to 40%

depending upon the way the manufacturer uses their imaging lens system. Steve Chapman protrain@emcourses.com Wed Feb 10

SEM:
Carbon steel boundaries

Someone from our Mechanical Engineering department requested me to take a look on the grain boundaries of her low carbon steel sample. I haven't viewed carbon steel before but I have a metallography manual somewhere and from it I used the (10 ml HCl + 5ml HNO_3 + 85 ml ethanol) etchant. Light repolishing was done after etching but I still couldn't get a clearer surface for image analysis. Suggestions? ***Melina Miralles mmiralles@pi.ac.ae Tue Feb 9***

We review dozens of steel samples daily as a process control for our heat treating operations (several processes—carburizing, nitriding, and through-hardening of steels). Our routine procedure is to polish through 1 micron diamond and then etch for 5 seconds ± with 5–10% HNO_3 in methanol. Rick Ross richard.ross@allison-transmission.com Tue Feb 9

You are using too strong etchant. Already mentioned nital usually works fine. Do not repolish after etching! Vladimir M. Dusevich dusevichv@umkc.edu Tue Feb 9

SEM:
imaging sucrose particles

I am collecting sucrose particles on an aluminum plate and imaging them with a SEM. I'm fairly new to the process and am having trouble finding any particles. I calculated that there should be about 23 particles per field at the concentrations generated for the test. I am able to bring the image into sharp focus, so I should be able to see any particles in the field of view. I'd like to rule out the possibility that the reason for this problem is that the particles are evaporating under the electron beam when I try to image them. The particles are 15 nm and I am imaging them at 65,000× at 15.0 kV. The particles also contain trace amounts of ammonium acetate from the buffer solution used while creating them. The sample is sputter-coated with 5 nm of gold prior to imaging. Should these conditions cause the particles to evaporate before I can focus on them? ***Daniel Thayer kanawai@hotmail.com Thu Feb 4***

How do you know you're in sharp focus? Unless this is an FESEM, 65k× is fairly high magnification for 15 kV. Are you sure you're resolving 15 nm? In fact you probably need to be resolving 10 nm or better to have a good idea if you're seeing your particles. What is your condenser lens/probe size/beam current setting? If your image isn't crisp (due to spot size, not focus), you might possibly have enough beam current to evaporate your sample. Is there a chance that you haven't actually made particles? Without a gold coating, they would be very hard to see. I'm assuming that you're gold coating is in the range that you describe. You might try 10 nm and see if anything changes. Ken Converse kenconverse@qualityimages.biz Thu Feb 4

15 nm × 65,000 = 975,000 nm = 975 microns = 0.975 mm. Basically, your 15 nm particle is a speck on the screen. Next, you are uniformly coating the specks and substrate with gold, at 1/3 the speck size. It is not surprising that you do not see anything. Especially, since you are using 15 kV on a light-element (sucrose) particle. The scattering volume dwarfs the speck size. Jim Quinn jquinn@www.matscieng.sunysb.edu Thu Feb 4

SEM:
flattening tissues

I was reminded by a recent posting (flattening samples for embedding) of a problem we have recently encountered. A student is trying to do SEM on the distal ends of critical point dried hummingbird tongues. The main body of the tongue is about 1 mm wide by 1–2 cm long, and bifurcates towards the end into two flat ribbons of tissue that form spirals during critical point drying (CPD), even if they are kept flat during the fixation and dehydration. We would like to force them to stay flat all the way through the critical point drying. Any sheets of material used to sandwich them would have to be rigid enough to prevent curling (possibly by mounting in a frame) but not mechanically damage the surface, and it would need to be stable chemically and allow adequate exchange during critical point drying. I would be interested in any experiences you have with different materials for creating such a sandwich (e.g., metal or polymer meshes, filter papers). I am also open to other ideas, but would prefer not to pin them, as it is likely to damage critical parts. Our protocol involves aldehyde and osmium fixation, followed by dehydration to 100% ethanol and critical point drying. No other solvents (besides the CO_2) are used. ***Marie E. Cantino marie.cantino@uconn.edu Fri Feb 19***

I have had some success preventing though not minimizing curling of plant tissues by encasing them between Formvar films. I make a wire loop, plunge it over a Formvar rectangle, let dry, put my sample flat on the film (when the sample is still hydrated) and then plunge again over a second rectangle. Then the loop + Formvar sandwiched sample can be dehydrated and CPD is no problem. When it comes time to mount on a stub you have to be a little dexterous and tease apart the Formvar films but this is not so difficult with practice. Everything gets through Formvar and the only thing I have found to dissolve it is propylene oxide. Tobias Baskin baskin@bio.umass.edu Fri Feb 19

This problem is really hard. During and after CPD even the slightest forces applied to the biological sample will destroy the fine surface details. When I CPD epithelial tissue, very often I find areas with marks created by the basket mesh when the sample hits against it during flushing with CO_2. So I think that any attempt to sandwich your sample the bird tongue- will cause extensive damage on it. What can you do then . . . I can only offer you ideas: 1. Cut with fine scissors the tongue in several cuts perpendicular to its long axis. The cuts should be partial, leaving one end still attached (looking like a single strand DNA in textbooks) so you will not lose the anatomy and orientation. Then after CPD—do it immediately, as the sample is still a bit elastic and not completely rigid—using fine forceps, very carefully, align the sample. Avoid touching the surface but touch the cut ends so the damage from forceps will not affect the surface. 2. Another idea (may be practically impossible but will be great if it works!). Try to insert a pin (can be fine needle or some plant spike or similar) starting from the basis of the tongue and follow the longitudinal axis towards the end and try to enter one bifurcation as far as possible. The idea is that an internal skeleton will keep the tissue straight without affecting the epithelium. But maybe this can also result to artifacts due to epithelial traction, I don't really know. Yorgos Nikas eikonika@otenet.gr Sat Feb 20

I apologize if I blinked and someone recommended this before, but how about making a replica of the tongue surface? Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Mon Feb 22

Here is a brief synopsis of suggestions, some of which did not appear on the listserver, for flattening our hummingbird tongues - make a surface replica - use HMDS - use cryoSEM or ESEM - pin or wire the sample to a steel mesh rack at intervals along its length - make partial horizontal cuts perpendicular to the long axis to relieve stress during drying, then realign just after CPD when the tissue is still elastic - insert a minuten pin through the tissue along the long axis of the tongue - cut into pieces, then reassemble on the stub after drying - process in tiny envelopes or sandwiches made from polycarbonate

filters, filter paper, Kimwipes, Formvar, window screen or biopsy bag material that has been stapled or sewn together We hope to test at least a couple of these ideas. Marie E. Cantino marie.cantino@uconn.edu Tue Feb 23

Not to add to the confusion, but I have been thinking about this for a few days and what bothers me is that the tongues do not curl during fixation and solvent dehydration, but do curl following CO_2 CPD. This suggests to me that that the CPD processing is at fault, and when I have seen similar things in my lab I always suspect improper CPD processing. My best test samples have always been 2% agar blocks (i.e., 2% agar 98% water/solvent/empty space)—if they come out of the CPD changed from when they went in then the CPD was not done right. I have had aldehyde fixation and solvent dehydration all produce structural stresses and changes most typically and causing curling etc. in tissues. Because of differential size changes fixation rates structural water removal etc. Problems with CPD: water left in tissue - incomplete dehydration. Failure to completely flush the solvent with CO_2. Water in the CO_2; I think as has been discussed on this list this is very rare. Failure to reach critical point. But most common I have found is failure to leave a gaseous head space following the last CO_2 flush. You need to leave a 20–30% (volume) head space in the CPD chamber. So you have a cold chamber (I really like 4–5°C) with 70–80% filled with liquid CO_2 and 30-20% gaseous CO_2, then isolate the chamber and raise the temperature (which will raise the pressure) until critical point is reached (or passed). And then vent very slowly. Richard E. Edelmann edelmare@muohio.edu Wed Feb 24

Since a few folks have asked me "Why the headspace?" I thought I'd answer. I am not sure as to why it works. We discovered it when I was a grad student. We were having problems with dehydration artifacts following CPD and one of us went back to the original early 1970's CPD paper and they very specifically said that the head space was needed for it to work. We tried it and everything was good! Richard E. Edelmann edelmare@muohio.edu Wed Feb 24

Ion Beam Milling:
gun lifetimes

Does anyone know the ion gun lifetime of: JEOL Cross Section Polisher SM-09010 Hitachi Ion Milling System E-3500? ***Andres Noah optik.a@a1.net Thu Feb 11***

I'm in charge of a JEOL cross section polisher. We bought the polisher three years ago (roughly 400 samples ion polished) and we haven't changed any part of the gun. However the gun (and the chamber) has to be carefully cleaned every 10–15 samples in order to get rid of the contamination. It takes usually one hour to clean it with fine polishing paper and ethanol. One electrode of the gun is prone to contamination and can be seen as a consumable, but its life time is long enough if it is not damaged during the cleaning process. In fact, it is mainly the shield plate that protects the sample that is a concern because it has to be changed every 20–40 samples and is expensive. Patrick Weisbecker weis183@yahoo.fr Fri Feb 12

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