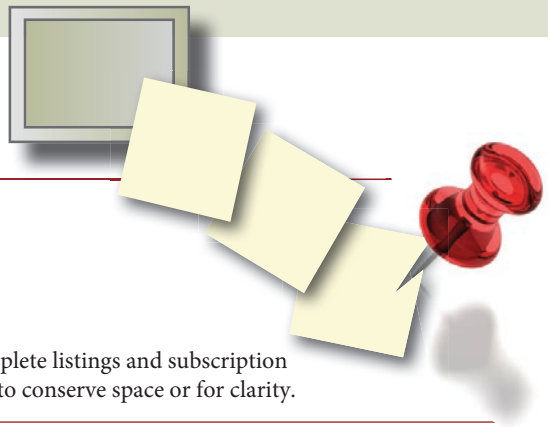


NetNotes

Edited by Thomas E. Phillips
University of Missouri

phillipst@missouri.edu

Selected postings from the Microscopy Listserver from March 1, 2013 to April 30, 2013. 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:
heparin

Will the presence of heparin in the buffer used to clear blood cause any problems with fixation or subsequent immunolabeling? This will involve brain tissue. ***Randy Tindall tindallr@missouri.edu Mon Apr 1***

Extremely unlikely to have an effect in my opinion. Heparin is a highly charged, sulfated glycosaminoglycan so I suppose the increase number of negative charges could increase non-specific background. Tom Phillips phillipst@missouri.edu Mon Apr 1

I don't think heparin will be an issue. Unless—the primary antibody would be directed against a component that is heparin-like, then you might expect some labeling of the blood vessel walls. Heparin's negative charge at physiological pH is not likely to give non-specific background as any heparin left behind after perfusion would repulse negatively charged gold conjugates. Jan Leunissen leunissen@aurion.nl Mon Apr 1

Specimen Preparation:
cytoskeleton

Is there a preferred fixative for the preservation of the filaments that comprise the cytoskeleton? Are there any techniques or procedures for improving the visualization of the filaments? Here at UNMC I have recently had two unrelated projects arrive where part of the goal is to see the cytoskeleton filaments. In one, the tissue is cartilage growth plate chondrocyte cells from mice, and in the other it is corpus luteal cells from bovine ovaries. There may be a third project in the future. The past few years the fad has been autophagosomes; I don't know if this is some new interest of the moment. Anyway, since I'm just a one-person core facility I would appreciate all possible advice. Thanks to all for your past help and I look forward to your future help and advice. ***Tom Bargar tbargar@unmc.edu Mon Apr 1***

Usually, the standard fixation in 2.5% glutaraldehyde with 0.1M phosphate buffer followed by 1% osmium tetroxide will work fine. A secret will be to cut your sections a little thicker than usual. For seeing cytoskeletal fibers, I like to cut dark gold sections. This holds true for following endoplasmic reticulum and vesicles as well. You will be able to follow them more easily in thicker sections. Use epoxy rather than LR White or Lowicryls, as the added density of the medium will give you better images. Also, stay away from Spurr's; again, you will not like the images. I like a combination of Ladd's LX-112 with DDSA, NMA and DMP-30 as follows: 47 mL LX-112 epoxy resin + 25 mL specially distilled DDSA + 28mL NMA (Nadic Methyl Anhydride). Mix these three components for 1 minute by hand in a plastic specimen cup, add a magnetic stir bar, cover the cup and stir at high speed for at least 30 minutes. Do not spin fast enough to make air bubbles in the mix! After stirring this mix, add: 1.25 mL DMP-30. Use a disposable plastic pipette to add the DMP-30 to the mix. Stir the DMP-30 activator thoroughly into the embedding mix by hand, then use the magnetic stirrer to stir the complete mix for at least 30 minutes more. Each routine E.M. sample requires about 24 mL of complete medium. More difficult to infiltrate samples require more embedding mix changes, and longer infiltration. Use propylene oxide as your intermediate fluid between your dehydrating fluid and your embedding mix. You can consider using reduced osmium (1% osmium tetroxide containing 1.5% potassium ferricyanide) in place of the 1% osmium tetroxide in your fixation procedure for added contrast. Mix these components immediately before using, protect this mix from room light, and rinse your tissue well with water after fixation and before dehydration. You can also en bloc stain with uranyl acetate. Following water rinsing after osmication, en bloc stain with 1.5% uranyl acetate in 0.1M sodium acetate buffer, pH 6.3 in the dark at 4°C, for 1–2 hours. Follow this with thorough water rinsing before dehydration. Edward Haller ehaller@health.usf.edu Mon Apr 1

I guess it gets down to what you mean by filament. Microtubules are sensitive to temperature—low temperatures cause them to depolymerize so it is important to fix at room temp at least. Lots of fixatives include calcium to stabilize membranes but I seem to remember some papers suggesting that calcium was also detrimental to microtubule stability but I am less sure on that one. Intermediate filaments generally survive a lot so are probably less temperamental. Tom Phillips phillipst@missouri.edu Mon Apr 1

Microtubules are, indeed, sensitive to cold, so fix at room temp. I used to have to decalcify crustacean skeleton with EDTA in with my fixative, and the microtubules came out looking great! Other filaments are more stable, although sometimes elastin gets faint. Tina (Weatherby) Carvalho tina@pbrc.hawaii.edu Mon Apr 1

Specimen Preparation:
bacteriophage

How does one process bacteriophage for SEM? Will osmium treatment help in SEM imaging? ***Ravi Thakkar ravi.thakkar369@gmail.com Mon Apr 22***

What I did was just to pretend I was doing a negative stain for TEM, without the negative stain. Deposit the viri on a coated TEM grid, remove the fluid, a quick sputter coater (quick), or none. This was for a small dodecahedral virus that attacked blue-green bacteria. This is assuming you have a low-voltage FE-SEM. Fixation and a quick coat might be more necessary if you are using a tungsten filament SEM. Osmium might help, but I haven't tried it. OsO_4 vapor fix should do. It also helps if your phages are attached to their victim's flagellae or pili. It makes a neater image, anyway. Phil Oshel oshel1pe@cmich.edu Thu Apr 25

Specimen Preparation:
infiltration problems

Kind of a perplexing problem here: we are having difficulty with good infiltration of intestine and pancreas tissue samples. After one bad run, we greatly extended our infiltration steps (Epon/Spurr's resin) and had a repeat of bad results. We use microwave fixation and infiltration and it has been consistent and reliable with almost all specimens we have used it. In the current problematic batches, a couple samples infiltrated

fine, while others in the same run did not. Interestingly enough, the control set of one tissue (intestine) infiltrated poorly, while the treated sets looked fine. In the pancreas, the treated tissue infiltrated poorly, while the control was good. Both sets were put through eight (8!) changes of pure resin, including of course some overnighters. We are going to keep banging away at this, but does anyone have any sudden flashes of insight? You know, the "Hey that happened to me, too, and this is what we did!" sort? ***Randy Tindall tindallr@missouri.edu Tue Apr 9***

I had some problems with pancreas last year. Some areas of the tissue block just crumbled. I use a standard Epon equivalent resin and standard ethanol dehydration, propylene oxide infiltration protocol. Eventually I decided that the major problem was related to fixation. Subsequently I personally made up the 2% formaldehyde, 2% glutaraldehyde in 0.1M PO4 and attended the perfusion so I could mince the tissue into 1mm blocks as soon as possible. I left the samples in fix overnight or longer. The results were much better. I do not recall a problem with intestinal tissue but that has been many years ago. Larry Ackerman Larry.Ackerman@ucsf.edu Tue Apr 9

Three people have now suggested that the uneven fields in our microwaves may be the culprit in our strange infiltration problems, so it is time to get serious about checking this out. We use Pelco Biowaves, which have been consistently reliable workhorses for years, with the Coldspot water recirculator. We'll do some testing with neon bulbs and check for bubbles in the water bath, etc. Randy Tindall tindallr@missouri.edu Wed Apr 10

Specimen Preparation:

stain contamination

I've recently experienced a contamination on my sections that doesn't look like the typical uranyl acetate or lead citrate precipitates. Rather it looks like large thin flake like particles. I use disposable 0.22-micron filters that fit on a syringe for filtering my stains. I'm assuming it is possible to get contaminate particles from filters. I would like to hear from anyone who has dealt with contaminants from filters. I can't attach a photo to this request, but contact me by regular e-mail and I can share an image if that would help in determining the problem. ***Tom Bargar tbargar@unmc.edu Thu Apr 4***

There are so many places your contamination can originate from! Have you tried looking at your sections without any stain to check if the contamination is present before the staining process? It would be good to rule out that particular problem. You can inherit contamination from your boat water, eyelash manipulator, forceps, and just from any dusty air circulating. When I see detritus on my sections, which doesn't look like stain contaminate, I usually thoroughly clean everything in my microtome room starting with my forceps, manipulator soaking my diamond knife in double distilled H_2O before cleaning it. Good luck! We've all been there! Pat Kysar pekysar@ucdavis.edu Thu Apr 4

I'll be happy to take a look at your picture. We sometimes (often) see contamination that looks like flakes—they often resemble the little things that make up the "dust" on butterfly wings, if you have ever seen something like that. I'm not sure where it comes from, but we sometimes refer to it as boat contamination. When we find it, we clean our diamond knives again, make sure our boat water is clean by changing the syringe and filters we use for filling them. Sometimes that helps. Sometimes not. Randy Tindall tindallr@missouri.edu Thu Apr 4

Imaging:

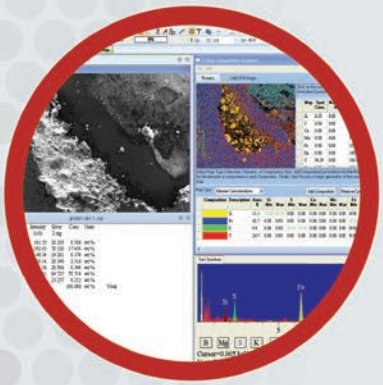
pseudocoloring for SEM

For some years I have worked on a new concept of visualizing specimen in the scanning electron microscope. My profession as a photographer leads me first to search for scientifically correct but also aesthetically appealing images, which makes me believe, that the scientific community and with it every researcher should be committed to share the aesthetics of the microworld with as many people as possible. In scanning electron microscopes (SEMs), data is usually shown using grayscale images. Colors can be added easily with additional detector signals. This procedure is widely accepted for communicating results on up-to-date research to newspapers, magazines and TV broadcasting outside the scientific world. In the past mostly still shots of the specimen had been shown or noisy TV rate screen captures. I thought it would be worth to bring the effects of a good movie into the SEM: fluent movement of the specimen, changing color and lighting effects during the sequence, changes of focus and magnification. Find out more at my website www.nanoflight.info. For those of you who like to see immediately, what`s this all about, go to www.nanoflight.info/nanoflight2.html directly and hope, that the server keeps running. I am open to any discussion. ***Stefan Diller stefan.diller@t-online.de Mon Apr 8***

Thank you for the links. The images and movies are fascinating indeed. I understand how important the appealing images are for the success in science: http://confocal-manawatu.pbworks.com/w/page/63945076/How%20to%20Be%20Successful%20in%20Science. However, I am doubtful about the scientific significance of the artificially colored images unless the color contrast represents variations or differences in physical/chemical properties where the fluorescence and Raman scattering microscopy or hyperspectral imaging for material characterization could be the examples. If we touch the technological base of the imaging as the source of the primary data for scientific knowledge: http://confocal-manawatu.pbworks.com/w/page/16347068/Technology%20of%20Research coloring the images (unless it leads to the production of new knowledge) is another technological operation. It adds time to the technological sequence of image acquisition and/or analysis and therefore reduces the efficiency of research. Unfortunately, I have a little experience in coloring of SEM images and I would appreciate your comments very much. Dmitry Sokolov dmitry.v.sokolov@gmail.com Mon Apr 8

I think the (Photoshop or similar) color on SEM micrographs once added helps later other people, including scientists, to distinguish structures that will take time to see without color. Nature loves camouflage; for instance sperm tails on the surface of a human egg (*zona pellucida*) are very similar to the structure of the *zona* and difficult to identify. Same is with cellular projections that spread over other cells etc. It could be an option for scientific journals to accept colored SEM micrographs if submitted along with the originals and checked for accuracy. Yorgos Nikas eikonika@otenet.gr Mon Apr 8

My Take: adding color can: a) add to the aesthetics, b) add contrast, and thus aid in recognition of morphology/structure/arrangement, c) add spatial separation and thus aid in recognition of morphology/structure/arrangement, d) add contrast or spatial separation and thus increase the speed of recognition (efficiency). Unfortunately, it can also distort (heighten or suppress) the underlying mathematical correlation (distance, shape, frequency), and even distract from the content. Note: We already accept distortions of phase into contrast (e.g., DIC [gray and color], PCM [gray], Hoffman [gray], etc.) or spatial position into color contrast [staining]; so the question is really one of reliability, relevance, reproducibility and transparent representation. There is also a sense of naturalness that might also be important. Think about the contrast effect of photographing a person's face with only light from the bottom up; it is much different than light from the top (natural sunlight) down, and thus the bottom up creates an eerie effect as a result of our human conditioning. 2. Marlana Coe's Comments (Human Factors for Technical Communicators) on color: Graphics, icons, and color perform vital functions: adds dimension, aids in decision making, enhances recall, focuses attention, renders



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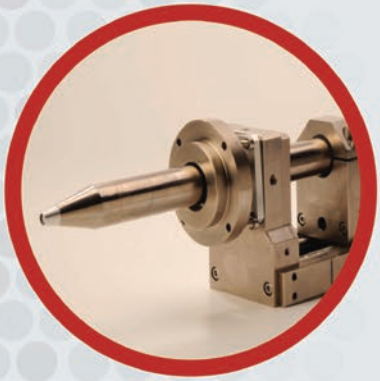

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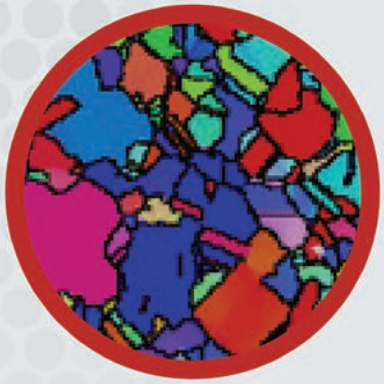

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images more realistic, reveals organization and pattern, satisfies users' preferences, speeds searches. She adds a couple of interesting comments: Users expect color in online information, but not necessarily in hardcopy. Hardcopy technical information is not enhanced by coloring the background or text. Users find it tiring, confusing, and distracting. Tony Tony's Refs: Burnham, "Color Perception in Small Test Fields," *JOSA*, 43, 10, 899–902, 1953; Burnham, "Comparison of Color Systems with Respect to Uniform Visual Spacing," *JOSA*, 39, 5, 387–391, 1949; Carter, "Color and conspicuousness," *JOSA*, 71, 6, 723–729, 1981; Caywood, "Independent Components of Color Natural Scenes Resemble V1 Neurons in Their Spatial and Color Tuning," *J Neurophysiol*, 91, 2859–2873, 2004; Cote, "Optical system performance visualization," *Proc 1999 SPIE Annual Conf*, SPIE, 1–12, 1999; Cramb, "Black and White versus Colour Photography," *JFSS*, 4, 2, 67–71, 1963; Wright, "Precision of Color Differences Derived from a Multidimensional Scaling Experiment," *JOSA*, 55, 12, 1650–1654, 1965; Mullen, "The contrast sensitivity of human colour vision to red-green and blue-yellow," *J Physiol*, 359, 381–400, 1985; Braje, "Human efficiency for recognizing and detecting low-pass filtered objects," *Vision Res*, 35, 21, 2955–2966, 1995; Fleury, Thesis, PhD, "Dynamic Scheme Selection in Image Coding," Lausanne, EPFL, 1999; Rubin, "Using color and grayscale images to teach histology to color-deficient medical students," *Anatomical Sci Educ*, 2, 2, 84–88, 2009; MacDonald & Luo, "Colour Imaging, Vision and Technology," 1999; Brockmann, "The unbearable distraction of color," *IEEE Trans Prof Commun*, 34, 3, 153–159, 1991. Tony Havics ph2@sprynet.com Mon Apr 8

But who decides what hue(s) are "natural" and "appropriate" for each structure? Different researchers might decide to use different colors to illustrate the same structure, depending on their own aesthetics and preconception. Differential coloring of the same structure in different publications has the potential to distract, distort and confuse. Also artificial coloring constitutes image manipulation. Once an investigator imparts structural boundaries using artificial color, it becomes more difficult if not impossible for others to visualize that image from a different perspective. That is, the audience can be easily led by the artificial rendering towards a particular line of interpretation or a particular way of quantification. This is not desirable for the sake of scientific objectiveness. To meet rigorous scientific journal standards both the original grayscale and the colored images need to be published side by side. Connie C.W. Hsia connie.hsia@utsouthwestern.edu Mon Apr 8

Clearly, the scientist has something in his mind when he takes microscopy photos and chooses his field, i.e. one or two out of the myriad aspects found in his specimen. The same is true for any researcher, whether he analyzes data or performs experiments and his results have the flavor of the way he had looked at things. By coloring your SEM photos you can finalize your results in a nice way and demonstrate what you had in your mind. In this sense the word "image manipulation" sounds bit dogmatic. But I agree that colored and uncolored photos could appear together. If your scientific results are images, everybody can clearly see and judge the "manipulation". Yorgos Nikas eikonika@otenet.gr Mon Apr 8

If something is "difficult to identify" it should not be colored! It can be misleading and manipulative. Vladimir M. Dusevich dusevichv@umkc.edu Mon Apr 8

Although I usually appreciate your comments very much, I don't agree on that one. Coloring a picture does not modify the content, it just make it easier to interpret. The data in all papers are interpreted by the authors; this is not a big secret. Actually, the authors even choose (wisely) the data to be presented! The objectivity of the choice made is in my opinion much more critical than the fact of coloring a picture. Especially for the field of microscopy where we usually can't display the results as statistical numbers, choosing the right picture to represent a result is critical. I think that it is good thing to present data in a way that makes them easily understandable. Stephane Nizets nizets2@yahoo.com Tue Apr 9

You have written "The data in all papers are interpreted by the authors; this is not a big secret. Actually the authors even choose (wisely) the data to be presented!" Unfortunately, for some of the papers "wisely" should be replaced on "wishfully." Electron microscopy data are prone to abuse (in most cases unintentional abuse). In my field, I tend to check presented data critically. Highlighting regions of low contrast with color can make this checking impossible and convince me (may be wrongfully) that data was manipulated. It's even worse if some features are highlighted and others, very similar, are left in gray levels. I have nothing against highlighting some obvious features, but why highlight something that stands apart anyway? Vladimir M. Dusevich dusevichv@umkc.edu Tue Apr 9

Imaging:

detection of elemental hydrogen or $Mg(OH)_2$ phase in magnesium oxide

I could use some ideas for the detection of hydrogen or $Mg(OH)_2$ on the surface of magnesium oxide metal surface. It seems that magnesium oxide will absorb water over time and this will interfere with a welding process. So to resolve the problem a preheat step is done to drive off the water. The results indicate this is not as effective as we think. So would like to detect components before and after heat treatment to see how effective we are. Any instrument techniques that could show us differences between treated and untreated areas would be useful. ***Roy Beavers rbeavers@mail.smu.edu Thu Mar 28***

My guess would be either of AFM techniques: scanning impedance, scanning capacitance, scanning spreading resistance, or scanning Kelvin probe microscopy. It still will be a matter of the actual properties of the surfaces pre- and after treatment. Dmitry Sokolov dmitry.v.sokolov@gmail.com Thu Mar 28

Electron diffraction from the surface of the sample might do it, depending on how the hydrogen or water adheres to the surface. Others are much more familiar with technical aspects of this technique, so perhaps one of them will also post. Bill Tivol wtivol@sbcglobal.net Thu Mar 28

I don't know about detecting hydrogen. It could be that infrared spectroscopy would detect a distinctive signal for the OH groups. The O:Mg ratio is 1 for MgO and 2 for $Mg(OH)_2$. I would think you should be able to detect that by EDS using low voltage to keep the excitation depth shallow so you see what is happening at the surface. Warren Straszheim wesaia@iastate.edu Thu Mar 28

Electron Microscopy:

electron emitter comparison

Our facility has instruments with each type of emitter (W, LaB_6, CCFE, and Schottky). We recently had a CCFE tip change, so I took the opportunity to image all of the emitters in both LM and SEM at identical magnifications for direct comparison. The images were also photographed both in and out of their respective caps. All of the images have been made into a PowerPoint presentation. If anyone would like a copy of this PowerPoint, please contact me and I would be happy to get the presentation to you. It is fairly large, so Dropbox would probably be the best way to ship it. ***Mark Grimson mark.grimson@ttu.edu Mon Mar 25***

Wow, huge response for the emitter PowerPoint! Maybe I should have charged for it! Too late now I guess! The file is about 84 Mb, so it is pretty big, but the images are 300 dpi, so Dropbox would be

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easiest, but I can always burn it on a CD and ship it that way. If anyone has any suggestions about alternative ways of sending this, it would be appreciated. I am not a heavy user of PowerPoint, so I may have overdone it on the size of the images. I am putting the finishing touches on the presentation, so it should be ready later today or tomorrow. Mark Grimson mark.grimson@ttu.edu Tue Mar 26

Pando is another possibility for up to 1GB. http://www.pando.com/what. Does TTU have an ftp site that anyone can download from? Just another thought. Your PowerPoint does sound interesting. Ken Converse kenconverse@qualityimages.biz Wed Mar 27 Mon Apr 1

Electron Microscopy:

introductory text

I am a first year Biochemistry and Molecular Biology graduate student and I am looking for an easy to understand paper on Electron Microscopy since I do not have much background in this technique. I would greatly appreciate any suggestions! ***Jetaime rossjeta@gmail.com Wed Apr 3***

I don't know of a paper but the best reference I know for beginning electron microscopy is the book by John Bozzola and Lonnie Russell titled *Electron Microscopy Principles and Techniques for Biologists*. It's a treasure of information beginning with a brief history of EM. The book covers specimen preparation for TEM and SEM, optics and theory of the electron microscope and much more. It's a volume I recommend to all beginning EM students. Pat Kysar pekysar@ucdavis.edu Wed Apr 3

Try hunting down a copy (in PDF) of: Introduction to the Scanning Electron Microscope Theory, Practice, & Procedures prepared by Michael Dunlap. Presented by the Facility for Advanced Instrumentation at University of California-Davis 1997. It is a 52-page concise overview for SEM. Tony Havics ph2@sprynet.com Wed Apr 3

One of the major EM manufacturers has produced a good short article: http://www.fei.com/resources/student-learning/introduction-to-electron-microscopy/intro.aspx You will find lots of information on the various types of light microscopy at http://microscopy.fsu.edu. Caroline Schooley schooley@mcn.org Wed Apr 3

SEM:

tilt calibration

With the move to a new lab and the installation of a new instrument, I have been performance testing all the microscopes. I also have an upcoming project, which will need some careful measurement of topography so need to know the tilt angle accuracy and repeatability. I thought no problem since I have circles on a Geller MRS-4 standard. A circle at tilt becomes an ellipse and one can use the ratio of max length/min width to calculate the perspective and camera angle, common in scientific photography. My math is sound because I can use the same standard on the light microscope with a known wedge angle and I measure correctly and am within 1 degree both with automated software measurements and by hand with Photoshop. But on the SEM, very carefully leveling the standard, correcting astigmatism, using a moderately fast scan rate to minimize drift errors, and taking into account the XY calibration ratios of the scan I am still seeing 5 or more degrees of variance. Is this the best I can expect or is there a better way to do this? ***Scott Whittaker whittaks@si.edu Wed Mar 13***

I would think that 5° would be really obvious in looking at the stage but if you're getting the angle from X vs. Y, that's going to be really touchy around 0°, so I'd suspect that your X and Y drives aren't stable (or consistent) or you are getting stage drift, thermal or otherwise in one axis or the other. I guess I'm saying that the stage tilt should be fairly accurate and reproducible at the 1° level, so I think the problem is elsewhere. See if the accuracy and/or reproducibility are better around 45°, where there is less sensitivity to X vs. Y because the changes are greater. One other thing to check would be the stage hysteresis for tilt. Come at 0° first from one direction, then the other and see how your measurements compare. I hope that there isn't 5° slop, but you never know. Ken Converse kenconverse@qualityimages.biz Wed Mar 13

What is the angle of your SEM detector that you are using to measure the circles? I believe that SEM detectors are at some angle to the specimen, probably even true for "in-lens detectors". This could account for the angle observed. Roseann Csencsits rcsencsits@lbl.gov Wed Mar 13

Thanks for the input. Indeed I have leveled the instrument, and drawn a line on the monitor of the chamber camera and can visually detect <1 degree tilt. Resetting the next run I used a bubble level on the instrument and matched another on the surface of the loaded sample to make sure it was as close to machine square as possible. In doing so I have now squared the machine. I am getting similar results from the other instrument and it seems even 1 pixel deviation in my measuring is significant, but since I can do this at the light level with just a penny on a known tilt I am confident in my measurement workflow on a traceable standard. For the next run I intend to make a set of self-referencing Teflon feet and run the stage up onto the lens to ensure the column/ stage is absolutely perpendicular and see if it makes a difference in my readings. This should square it, and help the mechanical drift I hope. I am somewhat mistrustful of anything until I can get 0 degrees to measure 0 degrees and I suppose this will be the reported error I use. Stage drift is definitely an issue, but based on earlier data I obtained measuring the drift rate during the performance testing the fast scan speed was chosen to be 1 pixel or less during capture at 968 lines. Image was noisy thus my decision to check the automated analysis with hand measurements via Photoshop. I had not considered thermal drift and just got all excited, but it seems that thermal drift would also be a part of the stage drift testing results and thus should have been part of the fast scan rate selection. I am out of ideas here and thank you for the valuable input. Who knew you could blow an entire day just checking the tilt angle reported on the stages. Scott Whittaker whittaks@si.edu Wed Mar 13

May you tell us more about your instrument? Motorized stage or not, model of the SEM? Do you use stereoscopy software? Nicolas Stephant micolas.stephant@univ-nantes.fr Thu Mar 14

Just out of curiosity, how much change in tilt does a one-pixel change give you? This would be a 0.1% error in linear measurement at 1000 lines. This level of accuracy is generally considered beyond the scope of SEMs unless it is a CD (critical dimension) instrument, although repeatability (precision) should be in that range, given identical working conditions such as working distance and a lens hysteresis procedure to normalize the lenses (and therefore the WD). When you're doing this with a light microscope, what are the critical parameters of the image you are working with? Is it simply from an NTSC camera, a high-resolution camera, or scanned from film? What kind of repeatability do you get? Try taking consecutive images of your standard on the SEM, then make all the changes and adjustments that you normally would except don't actually change the tilt. Take another image and see how it compares to the first two that were taken right after one another. Changes in lens hysteresis cause apparent changes in WD, which is a direct factor in calculating the magnification. If you focus, then go to a very long WD, refocus, take an image, go to a very short WD, refocus and take an image, I think you will find that the images may be identical, but the indicated mag will be quite different, or, if you set the mag the same, the images will be different sizes. This may be at least part of the problem with these very fine measurements. This is why a standard procedure for normalizing hysteresis is necessary. Some instruments have this built in (sometimes

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as an automated routine, sometimes as a lens reversal button), others don't. On older JEOLs and Amrays, there was a lens current reversal button. I found that pressing it for less than a second gave variable results, while pressing for a second or more gave much more consistent results. Newer JEOLs have a computer-controlled routine that is very consistent. On ETECs, the KV select button was undepressed, which turned off the lenses. I found that less that 15 seconds gave highly variable results. More than 15 seconds was more stable, but using the same time each time was important, so I always used 2 sweeps at the SYNC speed which was 16 seconds total and got consistent results for calibrating. It may be that you will want to use a different technique for measuring the topography in your upcoming project, if a high degree of accuracy is needed. On the other hand, if your image size (mag) is consistent, I would think that the stage tilt readout should give you enough accuracy for your stereo pairs and subsequent processing. Lots of variables here. Ken Converse kenconverse@qualityimages.biz Thu Mar 14

Would the tilt sensors like those in the MIAWiki page make your work faster next time?: http://confocal-manawatu.pbworks.com/w/page/64499329/Precision%20Tilt%20Sensors. The angular accuracy in XY plane seems being well below 1 degree. Dmitry Sokolov dmitry.v.sokolov@gmail.com Thu Mar 14

SEM:

vacuum level to avoid hydrocarbon contamination

I have an SEM equipped with a liquid helium cryostat that is used for CL spectroscopy at approximately 20K. It takes about 5 hours to reach 20K from 300K. It also takes around 4 hours to achieve ultimate vacuum in the chamber (9.6×10^{-5} Pa is the lower limit of the gauge). If I turn on the cryostat soon after loading the sample there is noticeable HC contamination that significantly alters the CL from the sample. Does anyone have an idea of what range of chamber vacuum would be appropriate for us to begin cooling the sample without having it behave like a cold trap? This would allow us to begin analysis in less than 9 hours, as is our current situation. ***Harvey Guthrey hguthrey@mines.edu Mar 7***

Based on the ungodly pumping time of 4 hours and level of hydrocarbon contamination that you are describing, chances are that your instrument is severely contaminated, with most likely sources of such contamination being non-vacuum-compatible oil used for lubricating state and/or poor-quality roughing pump oil back-streamed into main chamber. The way to resolve this would be to take apart vacuum system, thoroughly clean it with toluene followed by acetone + alcohol, discard all the O-rings and replace with new ones (Duniway Stockroom is supplying pre-baked O-rings, no interest—just a happy customer), religiously clean stage and lubricate it with y25-9 or better oil, install alumina trap on the roughing pump or use dry mechanical pump, and install Evactron or similar device to pre-clean chamber for each sample since no matter what you do to clean severely contaminated system oils will continue outgassing from crevices in the stage for quite a few years. If there is a diffusion pump, make sure that it uses oil compatible with UHV range, or better yet replace it with turbo. If you have spare ports on the chamber, you can use them to put in a couple of cold fingers to help trapping some of the oil molecules as a band-aid. Providing that you can't source hardware expertise to clean and dry the vacuum system and/or money to hire such expertise and pay for pump/trap/Evactron/cold fingers, then the only (known to me) realistic alternative is to wait patiently for system to reach ultimate vacuum and then start cooling, which you are doing already. Valery Ray vray@partbeamsystech.com Thu Mar 7

As Valery stated, the key factor is the partial pressure of hydrocarbons. These cause all the problems even when they contribute negligibly to the overall pressure. Cleaning the system and regularly using a de-contaminator (while costly) is the best way to eliminate the problem. Chamber plasma cleaners will allow even cryo-imaging, and even in non-UHV conditions. Larry Scipioni les@zsgenetics.com Fri Mar 8

EDS:

high resolution

Have you found significant inaccuracies with X-ray mapping when using a very high end FE-TEM (such as FEI Titan) and tilting the sample to the angle required for maximum X-ray counts? To my knowledge, the position of many EDS collimators requires a tilt of 20–30 degrees in order to obtain a sufficient count to offset background. The tilting does mean that the beam is traveling through a thicker and angled area of the sample. My concern would be in situations like mapping locations of molecules coating nano particles or other instances where very accurate location of elements relative to other nano features is desired. Does the tilting to this amount cause sufficient offset and location error to be a concern with very high-resolution instruments? How do you handle such situations if you do think it makes a significant difference? Are there some preferred detector/microscope criteria or combinations that reduce this problem and should be considered when purchasing an EDS system for this level of instrument? (Vendor comments appreciated but use discretion as to whether they should be offline). ***Debra Sherman dsherman@purdue.edu Thu Mar 14***

I use a Philips CM200 FEG. I have found the sample tilt is indeed very important in TEM/EDS quants. Even a tilt of only 20 degrees is a pretty small takeoff angle, and TEM samples are often not flat. Also, as you say, the spatial resolution of the TEM can make offset issues troublesome when measuring small grains. Also, make sure your k-factors are measured at the same tilt and beam conditions. I do the following, in order of importance, to get good quants: After centering your sample, and focusing, etc. Wait about 2–3 minutes for the stage to really stop drifting. Then make your measurement, or use a drift correction with a short turnaround (e.g. 20 seconds if you want to take spectra right after moving the goniometer). Some samples burn easily. Take a sequence of spectra each about 30 seconds long (or one minute, etc., depending on your beam intensity and count rate). Compare them. If they are different, your sample is changing! You can solve that by using a larger raster area or noticing that it is just one element that varies, and then use other methods to quantify it (I've used STXM, stoichiometry, backtracking a sequence of spectra, lower beam current, etc.) When the crystal is < about 200 nm in size, I always measure it using a map, with drift correction. Even for larger crystals, I often still use maps to quantify thickness variations, overlapping phases and chemical gradients. If it takes 20 minutes to get enough counts for your spectrum, then a 1-hour map covering 3× the area of your desired spectrum is sufficient to get the same counts. Then you can correct for nearby materials, hole spectrum, geometry, etc. I use homebrew software that applies k-factors and a thickness correction. I can tell it a thickness that I've measured using STXM or CBED or EELS, or if I can constrain it to a specific mineral system, then it will vary the thickness to optimize the stoichiometry (only possible with some minerals—long discussion in that). It turns out that even 100 nm thick specimens can have significant corrections for thickness—especially with small takeoff angles and elements < Si. So do not ignore thickness corrections, they can be very significant. Again, a map is sometimes necessary to get good estimations of actual absorption path lengths for the photons. When the crystal is about 100 nm thick, you want to make sure you are off a zone axis. This is mostly a problem with crystals that are at fixed orientation to the substrate. When you are on a zone axis, and the thickness of the crystal is on the order of an

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extinction length for one or another of the diffraction g-vectors, then you can get the ALCHEMI effect, which will alter your EDS by a few %. In practice this rarely happens—you have to get the alignment with a zone axis to be very fortuitous (or un-fortuitous), so you will not probably see it. Until one day, you have a spectrum from left field that makes no sense and you can't reproduce it (because you're not on the zone axis anymore). Zack Gainsforth zackg@berkeley.edu Thu Mar 14

EDX:

histological slide

I am planning to investigate the presence of micro- and submicro-sized aluminosilicate particles in rat organs. No labeling of the particles is possible, so I would rely solely on the optical detection of the particles (micro size) or on the analytical detection of Si/Al (submicro). The particles are not spherical, they don't have a definite shape so I expect the submicro-sized particles to be hard to detect in LM. I wondered if there would be some clever mind who could think of a method to detect Al/Si in histological slides (not stained, for this purpose). Perhaps I could simply put the slide in a SEM and do an EDX-mapping but I worry about the charging (glass slide) and it would be quite time-intensive. To avoid complications I would like to be able to detect the particles directly on a histological section placed on a glass slide in the usual way. I know no "histological" labeling of Al/Si based on chemistry but if someone knows better, I would be glad to hear about the method. ***Stephane Nizets nizets2@yahoo.com Thu Mar 21***

What you want to do is to have your histologist cut thicker paraffin sections. Lay one of these sections on top of a piece of double-sided carbon adhesive tape on your SEM sample holder, and remove the paraffin with xylene. Dehydrate the sample with 100% ethanol and allow the sample to air dry, then carbon-coat it. Use your back-scattered electron detector to locate your particles. Collect your secondary electron image and backscattered electron images of the sample before doing your EDS work, since the higher beam current for the EDS work will burn your tissue. After getting your photos of the tissue, do your EDS work, and you are finished. I've done this on pathology samples, and for forensic samples in the past. The technique works quite well. This works for finding ferruginous bodies in lung, for example, or talc particles, in the case of I.V. drug abuse. You definitely do not want to work from a glass slide. The nice thing about working with the carbon tape as a background is that the low atomic number background of the carbon tape will not introduce additional x-ray peaks into your spectra, and the tape helps ground your sample. I tried to work with sections 10–15 microns thick. Edward Haller ehaller@health.usf.edu Thu Mar 21

Two possible approaches: 1) Try polarizing light microscopy on stained and unstained sections. I suggest both, as it might be easier to locate the particles on unstained material but you may need the stained material for comparison to see their locations relative to other features. 2) Use EDS mapping with an SEM. Charging can be handled by either carbon coating the sample or using low vacuum (preferably with an EDS adapter on your microscope final lens. This acts to funnel the primary beam closer to the sample while protecting it from the low vacuum environs). To make mapping efficient, use an SDD system that will give you significant counts even at lower kVs (I would try 10kV initially) to get maps in acceptable time frames. Debby dsherman@purdue.edu Thu Mar 21

Thank you for your message, this is very interesting indeed. Seeing particles is very good, localizing them is even better. How can you localize the particles in relation to the histology with the method you gave me? I wonder what information the SEM can give? I will see wonderfully flat sections, no? Stephane Nizets nizets2@yahoo.com Thu Mar 21

In many cases, I would be able to do a direct comparison of my SEM image at low magnification with a histology photograph taken with the optical microscope at the same magnification, and find the same area in both images. In some cases, I would need to photograph the tissue still in the paraffin block and compare this image to my SEM section image. Essentially, you are working with serial sections from the same block, one stained with H&E, and one deparaffinized and carbon-coated, then photographed in the SEM by secondary electrons and backscattered electrons (2 images, one showing the particles by atomic number contrast). By photographing the area of interest at progressively higher magnification, you can document the area containing the particles, and then perform EDS to determine the particle makeup. Edward Haller ehaller@health.usf.edu Thu Mar 21

Great idea to mount sections on carbon tape. However, couldn't you do the same by mounting on plastic coverslips (thus eliminating the Si from the glass slides) and then be able to do SEM and LM on the same sections? If low vacuum was used without the carbon coating than it may be possible to stain the tissue after the SEM analysis, although I think prior staining might be okay as well depending on the stain. I haven't tried it so not sure on that. A main concern would be relocation of the particles during sample prep. Thicker sections (either thick paraffin or Vibratome sections) might reduce this possibility and would certainly be easier to work with and allow for excising high probability areas for TEM prep and analysis. Debby Sherman dsherman@purdue.edu Thu Mar 21

I had considered your idea, but don't think this would gain anything over using a glass substrate. I didn't, and still do not, have access to an LV SEM. Plastic coverslips will usually involve the use of some type of coating to hold the sections in place during de-paraffinization/dehydration, adding another step, and the plastic, being a non-conductor, will add to your problems with charging. Some plastic coverslips are pretty beam sensitive, and you can burn pits in them with the electron beam while collecting your EDS spectrum. All of these things can factor in to your sample prep choices. Your suggestion is feasible, but I don't think you will gain much over having serial sections, one H&E and one for the SEM, since these are only microns apart, essentially only a cell or two in thickness apart in the tissue. Edward Haller ehaller@health.usf.edu Thu Mar 21

http://www.jstor.org/stable/pdfplus/2442818.pdf?acceptTC=true Article: Detection of Silica in Plants. EDX/EDS is possible, but remember that x-ray emissions in SEM are generated by the action of the beam on the surface. Si and Al are light elements with relatively weak/low energy x-rays. Thus, the depth in the section of the particles will be a large determinant of their capacity to be detected by this method. Fred Monson fmonson@wcupa.edu Thu Mar 21

We all love to work with our SEM, but on second thought perhaps it is overkill. It seems that dark field LM would be appropriate to observe sub-microparticles with enormous advantages such as no special preparation needed and direct comparison with histology possible. http://www.ncbi.nlm.nih.gov/pubmed/16290430 I don't know if the paraffin embedding and the observation of thin sections can be an issue with this technique, though. Thank you for the interesting ideas and discussions. Stephane Nizets nizets2@yahoo.com Thu Mar 21

EDS:

quantification problem

We have an INCA X-sight EDS detector from Oxford Instruments and we have trouble quantifying the data. The detector collects the signals and software labels the elements while acquiring. However; when you want to quantify, it gives exactly this error: "No lines could be found for quantification of the following elements: Al, Cr, Co, Ni. This is probably due to the accelerating voltage being too low, quantification of

spectrum 1 aborted." Error 1940[4]. Although I rebooted both detector and microscope and changed the accelerating voltage, the problem wasn't solved. Has anyone seen this error before? ***Tugce Karakulak karakulaktugce@gmail.com Tue Apr 9***

Normally if my microscope is not communicating by RS-232 is when I get an error like this. I have a Zeiss system with my INCA. David M. David Frey freym2@rpi.edu Tue Apr 9

Typically, with an integrated system, Inca will read the beam kV from a SEM. It uses this information for quantification. Check to see if Inca is reading the proper beam kV. If it is not and the value it "thinks" you are using is too low, you will get the error condition. Not sure if it is possible to manually input the beam voltage used. I have never tried that. Woody woody@albe24.com Tue Apr 9

Whenever I ran into a problem that I could not easily solve I just called Oxford and got technical assistance. They are great about getting back to you quickly and helping you work through the problem. Don't hesitate to do this, as accessible tech help is one of Oxford's strengths. Debby Sherman dsherman@purdue.edu Tue Apr 9

Sounds to me like you haven't cleared your periodic table from your last analysis. Gary M. Easton garyeaston@scannerscorp.com Wed Apr 10

First check to see if the element list is fixed and has the correct elements in it or select current spectrum. If that does not solve the problem enter configure and select INCA auto select the lines as this feature maybe turned off. If this does not work, call Oxford. Keith Collins keith.collins@netl.doe.gov Wed Apr 10

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