



NetNotes

Edited by Thomas E. Phillips

University of Missouri

phillipst@missouri.edu

Selected postings from the Microscopy Listserv from November 1, 2014 to December 31, 2014. 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:

DNA stability on a grid

How long will DNA remain stable on a grid before rotary shadowing? Can I spread and collect the DNA by Kleinschmidt and the modified Kleinschmidt cytochrome C droplet method a week before coating without loss of detail? The purpose of the project is simply to identify if the strands are circular, branched, or linear. Not so important to have high resolution. Karen Kelley vau@ufl.edu Tue Dec 9

I regularly put DNA on carbon membranes with no over-coat protection - I have found them to be stable indefinitely, as far as their contour on the film is concerned. They are bound quite tightly - one usually cannot even wash them off! Larry Scipioni les@zsgenetics.com Tue Dec 9

Specimen Preparation:

quick fixation of cultured cells

I would like to have undergrad students quickly fix 3T3 culture cells growing on coverslips for SEM examination. I want the students to carry out these steps as part of an introduction to using the light microscopes, SEM, and TEM in a lab session where they photograph cultured cells labelled with fluorescent markers, cells on the SEM, and a slice of cultured cells or T4 negatively stained bacteriophage in the TEM. Unfortunately, there is very little time available for me to squeeze it into the lab session that would be available to the students. Furthermore, there are 140 students in the 2 hour 45 minute lab sessions (5-6 sessions over 3 days depending on enrollment), and only a small hood. I would like to avoid using osmium tetroxide because of the safety issues, and can't critical point dry that many samples (working in pairs is still almost 70 samples). A preliminary attempt, designed for speed and simplicity: Rinse cells in room temperature PBS. Fix in 4% formaldehyde, 2% glutaraldehyde in PBS for 10 minutes at room temperature. Rinse in room temperature PBS. Dehydrate in ethanol: 50%, 75%, 95%, 100% 3x, each @ 5 min. Air dry from 100% ethanol, or from HMDS (after 100% EtOH, 50:50 EtOH HMDS, 100% HMDS 2x each @ 5 min, then air dry). Cell are not happy in this preparation, looking either like outlines of where the cells were, or flattened and full of holes in their plasma membranes. Monitoring the cells suggests the problems are occurring after the start of the ethanol series. Any suggestions on ways for the students to better preserve these cells within the time and equipment constraints would be appreciated. Steven Barlow sbarlow@mail.sdsu.edu Tue Dec 9

When I've done cultured cells: Grow on coverslips - first sputter coat the coverslips on both sides for conductivity. 1 min max - this won't interfere with light microscopy. Fix with 1 - 1.25% glutaraldehyde in preferred buffer (0.1M phosphate worked fine) plus 1% monomeric tannic acid. The tannic acid helps with the membrane holes. But fix for 1 hour at room temperature. 10 minutes is too short. Dehydrate through EtOH starting at 30%, maybe lower depending on the cells 30-50-70-80-90-95-3x100 then to HMDS, but use more intermediate steps. 2:1, 1:1, 1:2 EtOH:HMDS, or even 3:1, 3:2, 1:1,

2:3, 1:3 EtOH:HMDS Then 3x100% HMDS. 5 min steps is generally enough if the cells are a monolayer, but I'd use 10 min for the transition to HMDS and the HMDS steps. Air dry either at room temperature or 60°C - which works better depends on the cells, just have to try. (Sometimes 37°C or 45°C is best.) This takes 1 hr or more. It's just possible that t-butyl alcohol would work. After the EtOH series, go 2:1, 1:1, 1:2 EtOH:tBuOH, 3x100% tBuOH, let solidify (freezes below 25°C) and vacuum sublimate. Tobias Baskin, May 2014 Microscopy Today. Leave just enough tBuOH to cover the cells before letting freeze. Also takes ~hr, maybe more. I have not tried this with cells, though. But, tBuOH is much less toxic than is HMDS. Not doable in one lab session. Phil Oshel oshel1pe@cmich.edu Wed Dec 10

Microscopy and Microanalysis 20, 1348-1355, 2014, "Extracellular Matrix Reorganization during Cryo Preparation for Scanning Electron Microscope Imaging of Staphylococcus aureus Biofilms" (doi: 10.1017/S143192761401277X). Comparison of Drying: In air; plunging in Liquid Nitrogen (LN₂), plunging in liquid ethane cooled in LN₂, and high pressure freezing in LN₂ - followed by low temperature sublimation of the frozen water. I suggest this to emphasize the value of high tech freezing methods followed by sublimation under vacuum, and cryo transfer equipment to move the specimen onto the SEM stage - itself held at regulated low temperatures. I lack these as well, so the article can provide a student with the difference between what s/he gets compared with what is now appropriate in the primary literature. I, and others, have used in sequence, OsO₄ gas (from 1 ml 10% OsO₄ in water + 4 ml water) to stabilize membranes in a closed "Ball" jar in the hood for 1-2hr; followed by an appropriate buffered fixative for 10-60min+ at 4°C. When I required a urinary bladder to be fixed at volume, I resorted to the following. Immerse bladder at volume in buffered paraldehyde for 10 minutes at 4°C. This poisons the musculo-neural system so that when the urine is released, the same volume of fixative can replace it to the same volume also at 4°C. I would then leave the bladder to fix overnight before continuing processing. The same works for TEM preps, but one must be aware that a rabbit urinary bladder wall becomes quite thin when distended. A colleague, and, as reported, colleagues of his, often used OsO₄ gas to stabilize his bacterial preps, either for thin sectioning or whole cell TEM studies such as negative staining. Fred Monson fmonson@wcupa.edu Wed Dec 10

Specimen Preparation:

fixing fern spores

I am looking for a protocol that can be used to fix Ceratopteris richardii fern spores for TEM. I am concerned about the ability of the fixative to penetrate the thick spore coat. I would also appreciate feedback and/or ideas about addressing this issue. Ashley Cannon ashleycannon@utexas.edu Tue Dec 30

Some years back I was working on ferns, two types actually. Some of the work that was not published was of the spores that had just cracked open and they were fixed well however I do not know if the

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fixative would work on intact spores. I do remember that the spore outer coat did not get well infiltrated and tended to separate from the Spurr's embedding medium. The fixative I used was 1% OsO₄, 1% glutaraldehyde, 0.05M phosphate buffer at pH 6.3 on ice and kept dark. It must be mixed just before use and it will be fine for an hour or so before oxidation can be detected and the solution will turn a light purple color. Tilney, L.G., T.J. Cooke, P.S. Connelly and M.S. Tilney, 1990. "The distribution of plasmodesmata and its relationship to morphogenesis in fern gametophytes," *Development* 110:1209–1221. For rabbit zygotes I have used DMSO to help the fixative penetrate and added acrolein to a glutaraldehyde and paraformaldehyde fixative but never tried it on spores. (acrolein is a chemical in tear gas - nasty!) Pat Connelly connellyps@nhlbi.nih.gov Tue Dec 30

Specimen Preparation: gold ring engraving

I have an antique gold wedding band (circa 1901) with an engraving on the inside that is quite well worn; some parts barely readable under an optical microscope. I mounted it in the SEM, with somewhat better results, but am not able to decipher the entire engraving. Is there any way I could enhance the engraving to make it more legible? I tried wiping it colloidal graphite and then immediately wiping it out lightly to see if the dag would settle in the depressions, but no luck. Gary M. Easton garyeaston@scannerscorp.com Mon Nov 10

I'd try grazing-incidence light. Have the illumination parallel to the metal surface. This will give the best chance of seeing surface topography. In the SEM, have you tried Y-modulation scanning with BSE in the topography mode? Phil Oshel oshel1pe@cmich.edu Mon Nov 10

Have you tried the side shot BSE using the SE detector? Just a thought. One other thought: The engraving should disturb the crystal structure of the gold, so it might be visible from an overhead BSE without etching the gold, as long as the general surface hasn't been smeared. Might be worth a try. Ken Converse kenconverse@quality-images.biz Mon Nov 10

You can try to obtain a replica of your surface. RepliSet F5 designed by Struers has an accuracy of 0.1 μ and can be observed by SEM after coating. Nicolas Stephant nicolas.stephant@univ-nantes.fr Wed Nov 12

Specimen Preparation: uneven polymerization

How can I correct the uneven polymerization of my resin in conical tip "bottleneck" BEEM capsules? The tips, where my pelleted cells are situated, are still soft, while the top of the block (base) is very hard. I know my mistake that I did not use a metal heat sink on the aspect of the tips. Can I continue to polymerize them in the 70°C oven with a heat sink without destroying interrelationships between organelles? They have been out of the oven a few days now, of course. Any other advice? Vickie Kimler vakimler@med.wayne.edu Mon Nov 17

You may have a closer look to Kent McDonald's paper in *Protoplasma* (2014) 251:429–448; he even suggested 100°C (!!!) for Epon + LR White, and did so, with all kinds of biological samples, with great success. No BEEM, though, if I remember correctly. Rachel Reinhard reinhard.rachel@biologie.uni-regensburg.de Tue Nov 18

I never had problems with polymerization at the tip of capsules. Your problem looks more like incomplete dehydration and since your material is pelleted, it affects only the tip. One way to verify this hypothesis is to cure the same capsule with the same resin but without material. If it polymerizes, then the problem is your material (probably incomplete dehydration). Stephane Nizets nizets2@yahoo.com Wed Dec 3

Microtomy: diamond knife angle

We have mistakenly ordered a Diatome 35° ultra diamond knife instead of a 45° ultra diamond knife and I am wondering if the 35° knife will be suitable for what I need? I particularly wish to cut high quality 80-250 nm thick sections of epoxy-embedded biological cells/tissues for TEM. Peta Clode peta.clode@uwa.edu.au Tue Nov 25

If you visit "<https://www.diatomeknives.com/>" (not knowing which brand you have purchased, Disclaimer: No affiliation, no financial Interest, only 34 years satisfied customer of these knives) you'll see that a diamond knife with 35° should be suitable for your task ("Ultra 35° the diamond knife for optimized sectioning results in almost all applications.") I personally have chosen in the 1980ies to use 45° diamond knives and always was happy with them. Unfortunately I don't have experience with 35° knives but I guess that sectioning will be as easy as with 45° (depending perhaps a little bit on the quality of resin [polymerization grade soft-medium-hard] you use). A [Diatome].pdf brochure you can find at: https://www.diatomeknives.com/knives/pdf/ultra_flyer_A4_USA_0306.pdf. There you can find: [Citation >>:] ultra 35°. In 1989 J. C. Jérior (Ref. Jérior) demonstrated considerably reduced compression, smoother section surfaces and improved structural preservation thanks to the use of our ultra 35° knives. In the meantime, a large number of scientists have recognized the advantages of 35° knives, in particular for sectioning Lowicryls and non-homogenous specimens, as well as non-decalcified bone, dental materials, etc. The ultra 35° knives are perfect for sectioning relatively soft materials research specimens including metals and polymers, as well as hard specimens such as semi-conductors, super conducting oxides, catalysts, nano-crystalline ceramics, etc. (Refs. Mahon, Glanvill, Swab, Quintana, Maniette, Schubert-Bischoff) [<< End of citation]. If you nevertheless are a bit hesitant about your knife's properties contact either your vendor for more information (brochures) or even contact Mr. Helmut Gnaegi at Diatome AG helmut.gnaegi@diatome.ch. He would be the best source of information I can imagine. Wolfgang Muss w.muss@salk.at Tue Nov 25

Image Processing: montage views

*I am stitching multiple digital images of paraffin sections photographed with either a 4× or 10× objective. A typical composite is 10-40 overlapping sections. Using my standard MagnaFire digital camera, I have used FIJI software to implement the background smoothing protocol outlined by George McNamara ("Color Balancing Histology Images for Presentations and Publication," *The Journal of Histotechnology* 28(2): 81–88, available at <http://home.earthlink.net/~geomcnamara/McNamara2005JoH28n2pp81-88.pdf>). Essentially you subtract a "black" image from all the images (to remove hot pixels) and then divide by a background white image (no tissue, blank spot on the slide) and multiply the resulting image by some factor such as 220. This works okay but is somewhat tedious. The method I really think does the best job employs Photoshop. I subtract the black image from every image just like in George's protocol and then use the Photoshop "Apply image - divide" using the background white image to divide the tissue image. It gives a great clean bright background. But this Photoshop function must be multiplying the divided image by something (and possibly doing other things) and, despite extensive online searching, I can find no documentation on what it is doing. I wouldn't try to publish an image where I have done an image processing step that I don't fully understand. Naturally I will describe what I have done in the Methods section - I have no worries on the ethical basis of what I am doing since I am going to explicitly state what I have done. But step one in that explanation is for me to understand what Photoshop does. Tom Phillips phillipst@missouri.edu Fri Nov 21*

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I took two images in which I drew a gradient; one was darkened so that brightest white was at 229 on an 8-bit scale. In the layers mode, you can also choose Divide. This divide is a straight up division, pixel by pixel: you can find blend mode formulas here: http://en.wikipedia.org/wiki/Blend_modes. The Layer Mode divide gave me an average reading of 229. The Apply Image - Divide also gave me the same reading. My suspicion is that it's something about your flat-field image that brightens the specimen image. You can prove this to yourself by brightening the flat-field image so that the brightest value maxes out at 255 (use Levels and the white slider until you see that the histogram's end is at max value), and then darken the flat-field image (use Levels and the output slider at bottom and type in 200 for max value). After division, you should see a difference in brightness values of neutral white background areas if the divide is a straight division, and it should correlate with brightness/darkness of flat-field image. Let me know your results. I don't have a lot of time now or I'd work this through numerically. You could do that by making an image at one tonal level and another at a darker tonal level and then divide one into the other and check with calculator for what should be the result. **Jerry Sedgewick** jerrysedgewick@gmail.com Fri Nov 21

I just tested one image that is at a value of 153 and a second at a value of 204. The resulting value after apply image - divide is 191. If 153 and 204 are divided by 255 to get decimal values, the result is .75. $0.75 * 255 = 191.25$. So divide is a straight division with no funny business. **Jerry Sedgewick** jerrysedgewick@gmail.com Fri Nov 21

The Apply Image - Divide is a pixel by pixel divide from what I've tested. It's the same as using Divide in the layer mode functions (http://en.wikipedia.org/wiki/Blend_modes). **Jerry Sedgewick** jerrysedgewick@gmail.com Fri Nov 21

Once you have divided by your illumination image (the one with no sample), your result is a ratio of your sample to a constant illumination, giving the fraction of light that your sample absorbs. Rescaling at that point is somewhat arbitrary, but the most common choices are to rescale by the scalar mean of the illumination image or possibly the scalar max. This is what is done in many software packages - Image Pro (our product, commercial disclaimer here) does this with the illumination mean. $\text{Image}' = [(\text{Image} - \text{BlackIm}) / \text{Illumination}] * \text{mean}(\text{Illumination})$. Or: $\text{Image}' = [(\text{Image} - \text{BlackIm}) / \text{Illumination}] * \text{max}(\text{Illumination})$. Either rescales the processed image into the same rough integer range as the original. Using the $\text{max}()$ gives brighter results, with less precision loss due to rounding with a brighter image overall, but risks saturation and clipping. Rescaling by $\text{mean}()$ is generally a safer choice. Again, the final result is somewhat arbitrary, but that doesn't really matter - even with perfectly flat illumination that didn't need correction (something I've never managed with a microscope) you can get different intensity final images just by changing the exposure time. What you gain with correction is a shift-invariant response of your sample. You can crosscheck the Photoshop method by doing these two alternatives by hand. If your step by step results match Photoshop, you've identified what scalar they use to bring the image back into integer range. Side note: you can do the same with fluorescent images, imaging a stained glass test slide (Chroma makes these) or a volume of dye in solution (gasket on a slide filled to slight excess, drop a cover slip on it, image mid-volume) to obtain your illumination image - thus characterizing spatial flatness for your microscope. **Kevin Ryan** kryan@mediacy.com Fri Nov 21

Thanks to Jerry and Kevin and everyone else who replied on my question. I don't know why I didn't think of doing the obvious thing and making a couple of homogeneous grey scale images and then dividing by some homogeneous brighter "grey" scale image and looking at the resulting pixels. To confirm Jerry's statement, I made a Photoshop image where I created some large bands of 120, 150, 175,

200 grey pixel values and then divided by various brighter images such as one whose pixels were all 220. The resulting bands were indeed the result of $x/220 * 255$. If $x >$ the denominator (i.e., brighter than the background white image), it causes saturation. **Tom Phillips** phillipst@missouri.edu Fri Nov 21

Subtracting the black image is performing a dark field correction. Hot pixels are commonly removed by a 3x3 median filter and persist after dark-field correction; just the brightest hot pixels will not be 255. You can simplify the workflow by averaging some representative dark-field images under conditions of a warmed up microscope and camera system, then measure the mean intensity and subtracting that from the working images. It probably won't change much from day to day unless your camera has high dark current and you use long exposure times. If you are using Fiji, the whole process from dark-field correction and flat-field correction to stitching can be automated with a macro or script. I've been writing some macros for mounting in our facility that might help you. Contact me off line if interested. Jerry, does Photoshop perform image calculations in 32-bit space and convert back to 8-bits, or does it stick with 8-bits? **Glen MacDonald** glenmac@u.washington.edu

Oh my. What you have described Photoshop doing is rescaling to the max integer value possible for the data type. That's probably OK for transmitted light, where you expect any sample to absorb and be darker than the flat field image, and as long as you ensure that your flat field image is taken with the same exposure time as your sample. But rescaling to the range of the image data type is wholly inappropriate for fluorescence, as the staining in your sample may or may not be brighter than your flat field reference. If you apply that Photoshop function to flat fielding fluorescence you'll have saturation/clipping of anything brighter than the reference. That's why IMO rescaling by the flat field mean is more appropriate - areas illuminated more than average get dimmed, areas illuminated less get amplified, all moving towards the sample image mid-range, and (on average) the overall intensity of the sample doesn't change under correction. **Kevin Ryan** kryan@mediacy.com Fri Nov 21

I am in full agreement that this would be wrong for fluorescence images since saturation can lead to an artifactual representation of the real image. My sections are all H&E stained paraffin sections and I am simply trying to illustrate the overall morphology of a large field of view. All the images, including the background image, were taken with identical exposures. Saturation of an RGB image of a paraffin section might risk losing detail but not lead to the type of misleading conclusion one would get while working with fluorescence images. One might avoid saturation by rescaling with a mean value but doesn't this lead to a compression since you are using less than the full 256 pixel range? **Tom Phillips** phillipst@missouri.edu Fri Nov 21

Rescaling by the mean will show histogram compression where the illumination is higher than average, and histogram expansion where it is lower than average. Scaling up gives only histogram expansion. But you're going to get ± 1 rounding losses for any scaling whether up or down (rounding direction being pretty much unbiased over the full intensity range and overall image) - I consider the absolute "loss" of data with saturation far worse than an integer rounding. And that's true even in RGB transmitted microscopy, as you risk losing dim edges of your objects to saturation, shifted automatic thresholds, and undercounted areas. Quite frankly, if my results (or perceptions) are conditional on a ± 1 intensity value or ± 1 pixel count, they are not strong results. **Kevin Ryan** kryan@mediacy.com Fri Nov 21

I am convinced. I guess it is a good thing I didn't think of the obvious use of test images to figure out how Photoshop worked because then I would have just proceeded with that strategy and never started this thread. I will give it a try with the mean value. I appreciate you

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taking the time to explain things so clearly. **Tom Phillips** phillipst@missouri.edu Fri Nov 21

Not sure if others have suggested this: ImageJ will give you much better control over an image than Photoshop will. <http://imagej.nih.gov/ij/>. The main advantage is that you can perform pixel operations that take the values outside the range of the original image (i.e., > 255 or even <0) and then normalize them later. This is really what you need for pixel operations and as far as I know Photoshop doesn't give you this degree of control. In addition to this I would recommend taking a look at Microsoft ICE for a free and really quick image mosaic stitching solution. <http://research.microsoft.com/en-us/um/redmond/groups/ivm/ICE/>. It's not ideal but it's quick and free, so may be worth running the images through this prior to trying the more tedious approach. Best results will come after doing the uneven illumination correction. **Ashley Norris** c.ash.norris@gmail.com Mon 12/8/2014 Fri Nov 21

Image Processing: diffraction image

I am getting a line in the diffraction pattern for all samples (<https://www.flickr.com/photos/97321550@N08/15858529368/>). I am sure this is not from the sample. It's something to do with scope. Can anybody tell me how to get rid of this line? **Ravi Thakkar** ravi.thakkar369@gmail.com Wed Dec 17

This looks like a TEM diffraction pattern with an incorrect timing on the blanking shutter. If you are using Digital Micrograph you can look under the Camera pallet, acquisition settings and set the camera to start acquiring the image 0.1 seconds or so after the blanking shutter turns off and see if it goes away. If so, then the explanation is likely that it was due to the image acquisition starting the instant the beam blanking turned off. Of course, during that instant the beam is still slewing back into place, so that faint line is actually the direct beam rastering back to its place under the beam blocker. Another hint is that if this is happening, the line appears stronger for shorter acquisitions and fainter for longer (though the actual intensity is the same if you count the number of counts). The time it takes for the shutter to turn off is fixed (say, 0.01 seconds of something) whereas the total acquisition time varies. A little caution, the shutter is often important to the well-being of your camera, so make sure you're configuring the right setting! **Zack Gainsforth** zackg@berkeley.edu Wed Dec 17

Image Processing: Hypermap and Qmap

Is there anybody using Bruker Esprit for elemental mapping in TEM? Could you please inspire me a bit about what's the difference between Mapping, HyperMap and QMap? Which one gives the real elemental content and distribution image? What I found is, after processing the Hypermaps using QMap, many tiny but shiny spots come out which actually don't make sense to me and the material. **Jason** 13qw9@queensu.ca Jason Tue Nov 18

When you take a HyperMap you save the whole EDX spectrum for each spatial pixel x, y to record the full spectral data cube. This allows you to process the data cube after it has been recorded, for instance creating maps from elements which you would not originally have chosen, pulling out line-scans or running the QMap (see later). If you instead take a Mapping you record only the integrated intensity for each chosen EDX peak, in other words acquiring a series of 2D map images rather than the full 3D data cube. Because you have to choose the elements to map before you acquire, and because you lose the spectral information which is important for checking that your maps are correct (for instance in STEM-EDX mapping it is easy to make fictive maps of background), and because you cannot do post-processing, I would never recommend this. Always record the HyperMap. Once you have acquired

the HyperMap you can then run a quantification (e.g. wt.% or at.%) of the data cube for chosen elements using the QMap function. Note that you can include elements for deconvolution without quantifying them, for instance to account for Cu signal from the grid. Whether or not the quantified maps give the correct elemental distribution depends on your specimen and on your point of view. If your specimen is a uniformly thick lamella and if you have enough counts per pixel that each pixel has a good quality spectrum then it should be good. However, if you have particles sitting on a carbon film or other objects of varying thickness then maybe not. This is for three reasons. First by calculating percentage composition you flatten the increase in elemental intensity that you associate with the object becoming thicker. Secondly small particles or objects may never give enough counts per pixel to give a spectrum that can be properly fitted and quantified (a situation that can sometimes be remediated by running a binned QMap). Thirdly the QMap will quantify every pixel, including for instance the carbon film and pixels in the vacuum. The latter two aspects differentiate STEM-EDX mapping from SEM-EDX mapping, which is typically done on bulk samples. They further have the effect of amplifying noise, since essentially you run noise peaks through quantification. This probably accounts for the shiny spots that you observe in your QMaps. To check for this, use the spectrum picker tool to look at the quality of the spectra for individual pixels and see if it is reasonable to fit a background and quantify them or if each spectrum actually has too few counts. As a final note, a "middle way" exists between the raw elemental maps – which are not background subtracted and which scale intensities between zero and maximum counts irrespective of whether there is 0.1 at.% or 99.9 at.% of an element – and the wt.% or at.% maps which have the drawbacks discussed above. This is to run the QMap and then ask to display the deconvolved, integrated peak intensities. If the data are noisy you can then follow this with a pixel smoothing. The resultant maps can give more truly comparative intensities while avoiding the "flattening" of intensity changes that are related to thickness changes. **Duncan Alexander** duncan.alexander@epfl.ch Tue Nov 18

About the differences between Mapping, HyperMap and QMap, have a look to the *"Man_QUANTAX_en.pdf"* that came with your Bruker system. There is there a clear description in a few lines. More interesting: the *"shiny spots come out which actually don't make sense to me and the material"* are most probably due to the use of the "Automatic Map Filter". This tool is not described in the documentation but I was told it contains a routine that adapt the filtering procedure/strength to the signal/noise in the map. On maps with a fairly high S/N it works well, but on noisy maps it groups some noise pixels and creates pseudo-nanoparticles that do not exist. Nice images, often meeting our expectancies... but wrong! This is in particular the case when you didn't accumulate enough counts in the Hypermap and QMap quantification warns you: *"Quantification: Very short acquisition time or low count rate. Quantification results may be inaccurate. Ignore or Cancel procedure!"*. There is a good chance if you choose "Ignore" that the quantification will be very inaccurate and your Automatic filter will build pseudo-nanoparticles that disappear if you choose no filter or another one. An advice: it's a good practice to always check how looks a spectrum extracted from the Hypermap over a square area of the same size as the QMap resolution (1: 1 × 1 = 1; 1/2: 2 × 2 = 4; 1/4: 4 × 4 = 16; 1/8: 8 × 8 = 64pxl; you read the area size on the left of the legend under the Hypermap when "Area unit" is set to pixels). Then you will judge how far can the Bremsstrahlung subtractions and quantification be accurate. **Philippe Buffat** philippe.buffat@epfl.ch Tue Nov 18

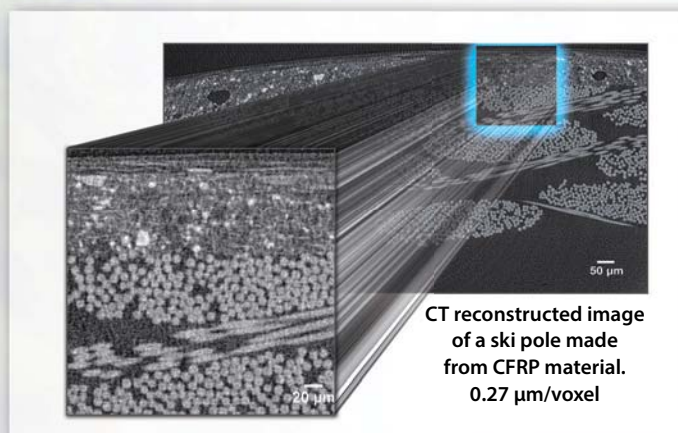
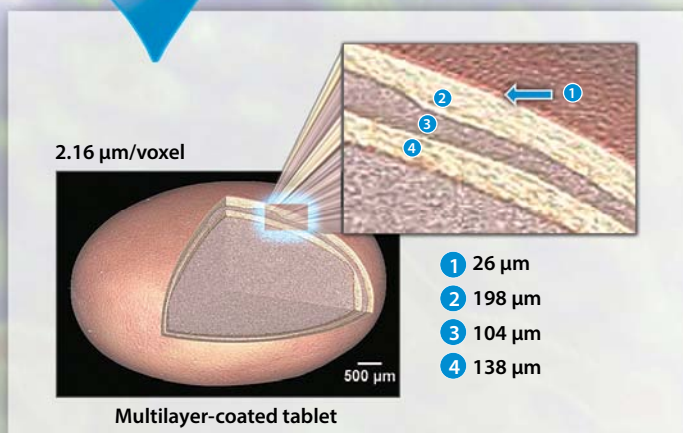
Image Processing: gamma settings in Gatan digital micrograph

I am interested in analyzing gold nanoparticles on an RBC surface. I have used Olympus AnalySIS before; it has gamma setting to visualize

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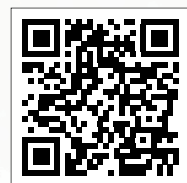
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nanoparticles on RBC surfaces. Does Gatan digital micrograph have such an option? **Ravi Thakkar ravi.thakkar369@gmail.com Thu Dec 11**

You'll find gamma, brightness and contrast sliders by selecting the menu item Window/Floating Windows/Display Control. ImageJ is a good place to do this as well (in my opinion a better place, actually). **Larry Scipioni les@zsgenetics.com Fri Dec 12**

Instrumentation:

vibration pad

We have a bad vibration problem in our building. Our old vibration pad has given up the ghost and is no longer made. Would like a recommendation for an anti vibration pad that can be up to four inches thick to place under the microtome to damp the vibrations, which are low frequency building vibrations. **Tom Reese treese@mbl.edu Mon Nov 10**

I've had some good success with Newport's VIBe series, which are easily configurable tables and they aren't as costly as the air-based tables. <https://www.newport.com/VIBe-Mechanical-Vibration-Isolator-Platforms/987419/1033/info.aspx>. For a really inexpensive solution - you can try the Meade or Celestron telescope vibration isolators - pack of 3 for about \$50. One of my customers used these under their Keyence digital scope and now they can get good images at 2000x - they have a cooling tower with big fan motors right outside on the roof near their lab. **Mike Toalson mike@materialanalyzers.com Mon Nov 10**

For another low-cost vibration-isolation solution, consider contacting your local monument making vendor and ordering a 12-inch thick granite plate of suitable dimensions. To isolate the granite place from the table vibrations use tennis balls from your local sports supply store. Quantity of tennis ball you will need depends on the mass of the granite plate and your instrument - try to have them compressed anywhere from 10% to 25% of the tennis ball diameter. **Valery Ray vray@partbeamsystech.com Tue Nov 11**

At the University of Pennsylvania I used a table constructed by using cinderblocks for the support legs and a huge cement slab that I think is commercially available as flooring in new buildings. This slab was approximately 4 inches thick but maybe 6 feet long. Cement held the whole thing together. A word of caution and that is to make sure that the table does not touch any walls or the small vibrations that flow down the walls will be transferred to your microtome. Valery's suggestion has the potential of being nicer to look at than the cement that I used and I have heard of using tennis balls previously but have not tried them myself. **Patricia Stranen Connelly connellyps@nhlbi.nih.gov Tue Nov 11**

Instrumentation:

Dewar vacuum fitting

I had a problem with a Noran Vantage Si(Li) detector. Many suggested the problem was due to ice, but I am reluctant to believe it. (You may check out an Excel file comparing various spectra or the EMSA files of the spectra at <ftp://ftp.marli.iastate.edu/Equipment/Noran-EDS>.) Anyway, we know the vacuum is quite soft in the Dewar and we need to do something. If the sorbent is saturated and we warm the detector up to deal with ice or send it back for repair, we risk blowing out the window and incurring a more expensive repair. Therefore, I wonder if we might beg, buy or borrow a fitting through which we could pump down the Dewar before and/or while we warm it up. It would be great if that alone solved the problem; however, I suspect we have other issues that will necessitate more serious refurbishing of the detector. **Warren Straszheim wesaia@iastate.edu Tue Nov 11**

When we had this problem in Albany, our shop made a back plate out of brass that had a copper tube with a Swagelok fitting on it. This allowed us to attach the Dewar to vacuum. We also from time to time removed the zeolite and either regenerated it or replaced it. We didn't have

a mate to the existing vacuum fitting on the Dewar, but our work-around functioned very well. **Bill Tivol wtivol@sbcglobal.net Tue Nov 11**

What is the procedure for removing the zeolite? I always assumed that it was somehow packaged inside the walls and therefore permanent. **John Twilley jtwilley@sprynet.com**

I cannot remember the set-up, since it has been well over a decade since I was in Albany. Perhaps someone from Noran (or whichever company bought them out) will be able to help. **Bill Tivol wtivol@sbcglobal.net Wed Nov 12**

Instrumentation:

liquid nitrogen filling system

I am looking for an automated liquid nitrogen (LN₂) filling/ transfer system for an EDS installed on scopes. Kindly share some suggestions based on your experience. **Ravi Thakkar ravi.thakkar369@gmail.com Mon Nov 17**

My advice is to forget about auto fill systems. The systems are unreliable. They either fail to turn on, which is a minor problem if your detector has a working bias shut-off (major if not), or worse, they fail to shut off, which empties the liquid nitrogen supply all over your microscope and floor and can cause serious damage. Also, any arrangement that gets them to not touch the microscope and transmit vibration exposes the Dewar to introduction of moisture, which induces ice crystals inside the Dewar, which degrades performance. **John Mardinly john.mardinly@asu.edu Wed Nov 19**

I am inclined to agree with John. I suggest you save the money you would spend on the auto fill system and keep saving to buy an SDD detector to get rid of the LN₂ completely (from the XEDS systems). They are not cheap but they are a lot less hassle than Si-Lis. **John Mansfield jfmjfm@umich.edu Wed Nov 19**

The benefits of going with SDD are much greater than just not having to deal with the LN₂. The systems are so much more sensitive and you get so many more counts that the time saved is an added bonus. They result in much more accurate results due to the high count rate, efficiency of the systems and more sophisticated software. While at Purdue, I justified replacing a 2-yr old SiLi system with the SDD to my administration partially on the safety considerations. A picture of a technician up on a stool pouring LN₂ into the Dewar and the obvious hazards involved made a strong argument for getting internal funding for the replacement. **Debby Sherman dsherman@purdue.edu Wed Nov 19**

Taking the cue from Debby's reply: is there anyone filling the Dewar in a better way than standing on a stool? I discussed the topic with NMR people here; they use a silicone tube connected directly to a LN₂ tank. Does anyone use a similar system? Does anyone see problems in this method? Maybe degradation of the material, falling into the XEDS Dewar? **Davide Cristofori dcristofori@unive.it Thu Nov 20**

The problem with the silicon tube method is that you first have to get the tube properly cooled down until no more gas but only liquid is transported. To control that initial phase safely immersing the tube directly to the EXDS Dewar might be even more difficult than externally filling first your small Dewar, away from the microscopy at a safe space, and then walk with the small Dewar to the XEDS Dewar via a stable stairway. I wouldn't be surprised if the initial strong gas flow directly going into the XEDS Dewar would even damage something in the XEDS Dewar on the long term. I would not spend too much time on thinking about an automatic filling system, but just think about how to replace your stool by a proper, stable, fixed-in-place stairway. **Marco Möller mmoller@cicbiomagune.es Fri Nov 21**

When we tried that at the HVEM, we had a problem when the system failed open circuit. This caused LN₂ to flow continually, and if someone had not entered the scope room for an unrelated reason late Friday, LN₂ would have continued to flow all weekend. The excess



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LN₂ caused the Dewar bottom to become convex, and our shop had to make a tool that we could use to return the Dewar to its proper shape. Fortunately, the instrument recovered and was usable for many years after the event. After that experience, we filled the Dewar manually every Friday--the LN₂ lasted more than a week in our set-up. **Bill Tivol** wtivol@sbcglobal.net Wed Nov 26

TEM: venting electron gun

I operate a Tecnai 12 Spirit transmission electron microscope. In order to replace the filament, you have to vent the electron gun (thermoionic), according to the FEI operating manual, before lifting up the gun cover. The problem is, when I vent the gun it doesn't vent completely to the atmospheric pressure (log 99), it keeps holding some vacuum (log 95) that prevents me from lifting up the gun cover to change the filament. Has anybody ever experienced anything like that? **Ahmad Ashkhaibi** ashkhaibi@yahoo.com Tue Nov 25

I don't have a Tecnai TEM but have a similar problem with our JEOL microprobe. The chamber needs about 1.2 vent cycles to come up to atmosphere. I push the vent button and wait, then press the evacuation button until the pumps start and then press vent again. We do have an FEI Quanta SEM. I think the software runs it through a vent cycle anytime we press vent. We found that there was about 20 seconds of extra venting whenever we vented our chamber. That chewed through extra tanks of N₂. The service engineer was able to reset the vent timer in the vacuum controls reduce the waste. I suppose a similar timer exists for the Tecnai in case you want to lengthen the time. **Warren Straszheim** wesaia@iastate.edu Tue Nov 25

It is possible that Pirani gauges are out of calibration. There will be a pot somewhere to tweak the gauge calibration - probably best to get a service engineer to check and do this. **Larry Stoter** larry.stoter@gmail.com Tue Nov 25

SEM: Wehnelt cup corrosion

Regarding our JEOL 6400 SEM and, particularly, its Wehnelt unit. On the three last times we exchanged the filament, we noticed that the Wehnelt cup at its outer surface hole (where the edge of the W filament is located inside) appeared to be like corroded or melt. The effect was more pronounced each time we removed the Wehnelt to exchange the filament. Furthermore, every new filament was placed into the center of the cup hole and the gun operates just below saturation conditions. Any advice why this is happening and about possible ways to prevent it will be highly appreciated. **Andreas Delimitis** andel@cperi.certh.gr Tue Nov 11

If what you're seeing looks like a peeling surface, then I think that you are not cleaning all of the plated tungsten off of the interior of the Wehnelt. When it peels, it changes the electrostatic field and causes imaging problems. Even if it doesn't peel, there is often gas trapped beneath it that will eventually release in small pulses, making your beam jump just a little each time. The trick is to inspect the Wehnelt very carefully after cleaning (with a light microscope) and reclean if necessary. **Ken Converse** kenconverse@qualityimages.biz Tue Nov 11

It seems you have flash-overs from the high voltage. Check your vacuum in the gun section. Do you have a stable imaging or does the image shift occasionally? **Stefan Diller** stefan.diller@t-online.de Tue Nov 11

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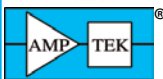
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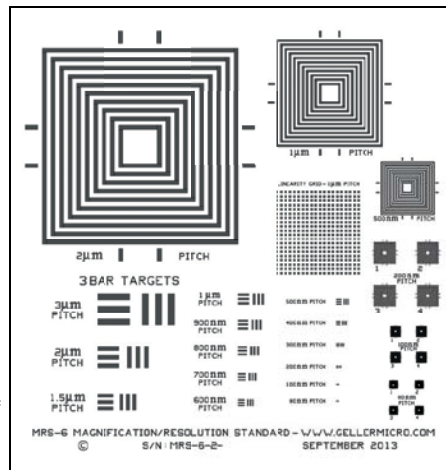
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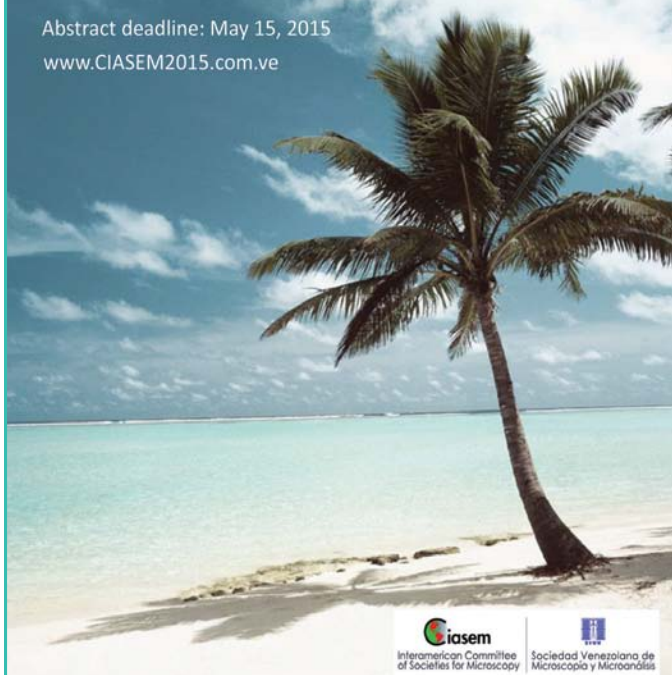
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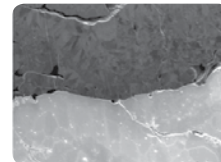
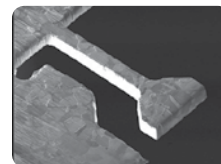
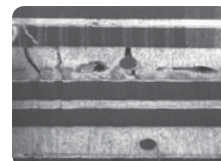
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