NETNOTES

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Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 10/10/04 to 12/10/04Postings may have been edited to conserve space or for clarity.

LM - Halogen warm-up period

I've recently started taking birefringence photographs of Congo Red stained tissue. I've noticed that over time, the very same field doesn't appear the same and that the labeled surface varies up to 25%. Everything being very stable (all screws firmly screwed, polarizer glued to its base), I'm wondering if anyone has performed a test to check the warm-up period of a halogen 6 volt lamp. The company who sells the lamp says 5 to 10 minutes, but could it be longer? Thank you! Marie-Claude Belanger <mcbelanger6@hotmail.com> 15 Oct 2004

I have some chemists doing spectroscopy using an inverted microscope base in our facility. Ours is a 12V halogen lamp, but their tests indicate a 4 hour warm up period for maximum stability. Having the lamp power supply rheostat adjusted to provide nearly highest output (highest color temperature) aids in warm up. Also, a new, high-quality bulb might help in your case. Karl Garsha <garsha@itg.uiuc.edu> 15 Oct 2004

We have found that many of the standard power supplies provided by the microscope manufacturers are noticeably unstable as measured with tube or CCD camera time lapse imaging of cells by phase contrast, at least with the power supply from the wall of 102 to 118 VAC here in NYC. On the microscope stations where we have needed to rectify this, we have purchased special stable power sources (AC to 12V DC conversions) instead of using the ones supplied in the bases of the microscopes. Michael Cammer <cammer@aecom.yu.edu> 15 Oct 2004

LM & EM - Water marks on negatives

I've been having problems with water marks being left on my micrographs once they have dried. Does anyone know of any way I can minimize this? Anna Young <Anna.Young@warwick.ac.uk> 03 Nov 2004

Most of the watermarks on negatives are a problem of too fast drying of the film or use of too little wetting agent (like PhotoFlo from Kodak) in coherence with calcareous washing water.. Normally - if the gelatine of the film is not disturbed with too hot drying - you can soak the film again, rub it cautiously and do a new drying cycle, best not above 100° F. If you are talking about watermarks on the prints, the problem is the same. Use wetting agent as last bath; use an infrared dryer or use a windscreen wiper blade to get rid of the water on both sides of the prints. Stefan Diller <diller@stefan-diller.com> 03 Nov 2004

I assume that you are washing your film in tap water and then drying. You need to give your film a final rinse with a few drops of detergent to act as a wetting agent. You can use ordinary washing liquid, but given the cost of film and time it's probably better just to stick with a commercial film wetting agent such as Kodak Photoflo or Ilford Ilfotol (if it's still available). I generally make up a tank of distilled water (rather than tap water) with a few drops of wetting agent for each film. Any decent photographic shop should supply a range of wetting agents. It's also possible to use special squeegee tongs to remove most of the water from film (especially 35mm) and further reduce drying marks, but if any grit ever gets onto the rubber pads it would cause more damage than drying marks. If you have old drying marks it may be possible to remove them from film provided that they aren't on the emulsion side. A nice long soak in distilled water and wetting agent can help, although I'm sure other microscopists may have some magic techniques of their own. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 03 Nov 2004

TEM - digital resolution equivalency of film

Could someone provide the digital equivalency (in terms of resolution) of 3.25 x 4 inch TEM film. I have read that a 6 megapixel digital file has the resolution capability of a 35 mm grayscale negative. If this is true, then an 18 megapixel digital file would be equivalent to a TEM grayscale negative in terms of resolution capability. I was thinking (based on darkroom enlargement capabilities of TEM films) that a 180 MP digital file would be more likely to exhibit the resolution one would see in a TEM negative. John J. Bozzola <bozzola@siu.edu> 18 Oct 2004

Film with a fine grain size and lots of silver (e.g., TEM film) can resolve the equivalent of about 4000 points per inch ("pixels" if you like). Good film scanners can digitize film with that resolution. That means your 3.25x4 inch film would represent 13000 x 16000 pixels or about 2 x 10^8 pixels. But it would take twice that many bytes to hold the data because the dynamic range of film greatly exceeds 8 bits. Film with a lot of silver in it can easily produce 12 bits (1 part in 4000) and even a bit more. A MaxD of 4.2, which is possible with X-ray film, corresponds to about 14 bits. So you would have to store the data as two bytes per pixel. Pay no attention to the myth that a 6 megapixel camera delivers the performance of film - it isn't close in either dynamic range or spatial resolution. John Russ <drjohnruss@aol.com> 19 Oct 2004

Digital equivalency depends on, first, the grain size, and, second, the scanner pixel size. For most film, the grain size is small compared to the pixel size; however, for pixel size less than ~5 µm, grain size may be important, and, as pixel size decreases below 5 µm, grain size becomes increasingly important. A 3.25 x 4 inch film is roughly 16,000 x 20,000 5 µm x 5 µm pixels for a size of ~335 Mpixel, so, if you have a good scanner, 18 Mpixel does not contain nearly as much info as there is on a scan of a negative, and, furthermore, the negative itself has about 1.5 orders of magnitude more information than is retrieved by a good scanner (and even more, if you take into account that the ODs in the film may not be scanned quantitatively). High-resolution film, such as 4489, has even better resolution than these figures would indicate due to its extremely fine grain and relatively good OD range; SO163 is pretty much in line with these figures; LoDose X-ray film is somewhat poorer. The drawback to 4489 is that it takes more beam to get an image, and LoDose requires much less beam, so, since radiation damage is often limiting, it is sometimes best to try for less information with less damage. There is no free lunch. The equivalence of 6 Mpixel with 35 mm film may have been derived from assuming a certain print size and using the highest spatial frequency seen by the human eye. In other words, comparing a print from a 6 Mpixel file with one made from film, one would see no difference. Bill Tivol <tivol@caltech.edu> 18 Oct 2004

I think, all of these calculations come to approximately the same numbers for the total information content of a negative, something of the order of one to several hundred MBytes. I think, you are also right in that the calculations yield MBit instead of MByte due to the binary nature of the silver grains. However, what we are talking of is the total information content of a negative. In the overwhelming number of cases, people don't need or don't even want this amount of information. Typical cases are Pathologists, who want to see the information on the entire negative, but don't care so much about the details embedded in the film at the grain level, or people who do high-resolution TEM. They are mostly interested in the highest resolution they can get, but not across the entire negative. In my opinion (as a vendor of digital systems), the digital cameras have already surpassed film in terms of usability and other parameters. The total information content of film is higher, but to me that's like a newspaper: Some people want to read the comics, others the stock listings, a third person might prefer the political or foreign news. On a day to day basis, nobody really needs all the information that is in the newspaper. And in the rare case that someone actually does need the full information content, you can always mosaic several TEM images into a large one. Mike Bode <mb@soft-imaging.com> 19 Oct 2004

TEM - Charge effect

We have a client looking at various incarnations of carbon (nanotubes, etc.) in the TEM and he is very curious about a phenomenon he is seeing. This involves dark bands traveling rapidly down lengths of carbon during viewing and is very similar to an effect I have seen commonly when looking at crystalline structures. I bet most, if not all, TEM users have noted something similar at some point, such as when looking at negatively stained specimens

when part of the stain has crystallized. When the beam hits a new area, there appears to be almost a liquid flow within the crystal which stabilizes in a few seconds. I have never given this much thought, assuming that this is just electron flow within the particles somehow affecting the beam to give this visual result. Can someone describe the physics, and possibly significance, of this? Randy Tindall <tindallr@missouri.edu> 29 Oct 2004

Diffraction contrast is very dependent on alignment, so when the lattice is oriented such that many reflections are excited which occurs, e.g., along a zone axis many electrons are scattered and the image looks dark. Alternatively, for orientations differing by only a few degrees, few reflections are at the Bragg angle, so there is little scattering, and the image looks light. Since the beam will heat the crystal locally and deform it especially if there are stresses that have been incorporated into the crystal during its formation, the orientation can change from one that is strongly scattering to one that is weakly scattering and vice versa, so dark and light bands will shift as the local orientations change, then stabilize as these orientations cease to change. These stripes are called bend contours, and they are discussed in many EM books. Without any guarantees, I guess that Electron Microscopy of Thin Crystals, by several authors of whom Howie and Whelan are two (I think), and Cowley's Diffraction Physics would discuss this and other topics of interest to your client. Bill Tivol <tivol@caltech.edu> 02 Nov 2004

FEG ESEM – Cryo vs. high vacuum

Some time back there was a discussion about the utility of an FEG ESEM. Specifically that the resolution under ESEM conditions was not all that good and that one would do just as well with a tungsten filament ESEM. Is this the prevailing opinion of FEG ESEM users? Are there too few instruments in service to form a consensus? Second, in terms of viewing hydrated biological samples and beam sensitive polymers and other materials, would one have better results with a cryo stage on a high vacuum FEG SEM? Greg Erdos <gwe@biotech.ufl.edu> 28 Oct 2004

Resolution in ESEM mode is close to resolution in high vacuum mode for "good" specimens (like Au particles). The problem is that resolution for uncoated specimens is very specimen dependent. I can get good pictures of Au particles at a magnification of x100k with FEG ESEM, but only at x20k for hydrated hard tissue, such as bone or dentin. I do not use magnifications above x30k for any specimens in ESEM mode, including minerals and ceramics. Hydrated soft tissues are usually covered with a thin layer of water which hides small details, so for them useful magnifications are considerably lower. As for beam sensitive specimens, I think the best results could be obtained with coating or in low voltage mode (and for low voltage, FEG is better). Vladimir Dusevich <dusevichv@umkc.edu> 28 Oct 2004

Correct me if I am wrong, but I believe the context for that previous consensus was not for "ESEM" (i.e., 2500 Pa), but rather for "VP-SEM" (i.e, 250 Pa) and that FEG was somewhat useful in the VP range of pressures. That is, you'll almost always find some utility in using a brighter gun, unless high pressure unduly contaminates the FEG source. Michael Shaffer <michael@shaffer.net> 28 Oct 2004

I cannot comment on a FEG ESEM since we do not have one at present. I have worked with a W-ESEM and an SEM with cryo-stage and came to the following conclusion based on samples I have used: 1) A great strength of ESEM is the ability to do dynamic experiments. In this case resolution is usually secondary. However, FEG should give better resolution than W- with appropriate samples. 2) Either ESEM or low-vacuum can be helpful for minimizing charging when coating is not desired but this usually compromises resolution when compared to high vacuum imaging. 3) Hydrated samples are difficult to work with under the best of circumstances and ESEM has its own challenges. 4) Cryo-SEM is the way to go for static hydrated imaging whenever possible. You can work with fractured samples, sublimate to remove unbound water, and work with delicate hydrated samples such as young plants with minimum problems. Resolution is normally acceptable for these types of samples

with preservation far superior to the samples prepped for high vacuum imaging. Charging is easily handled using coating under cryo conditions. 5) Cryo permits easy imaging of heat sensitive polymers and other delicate materials. They can be coated at low temperature if charging is a problem and resolution is not seriously compromised over standard high vacuum imaging. 6) Ultimately resolution depends much more on the sample than on the capabilities of the instrument. However, there is a greater potential to work at higher magnifications while retaining good resolution with the FEG and resulting increased beam coherence, smaller beam diameter, good signal:noise ratio, and ability to work at lower kV. My opinion is to go with FEG whenever possible providing you can afford the increased cost of the original instrument and the service contracts. An ESEM can still benefit from FEG in high-vacuum mode even if there may not be a great difference in ESEM mode. Although it might not be needed for many samples now, it will be helpful for some. You never know about future needs and most of us cannot justify replacing instruments on a frequent basis. Remember that cryo can be added to any SEM so you are not limiting that option with either ESEM or FESEM. Debby Sherman <dsherman@purdue.edu> 28 Oct 2004

SEM - Spatial frequency, noise and magnification

I need to know the relationship between spatial frequency, noise and SEM magnification. Sim Kok Swee <kssim@mmu.edu.my> 12 Nov 2004

It is inappropriate to use the same filter for the two different images. A filter should be tuned to the frequency content of the information contained in the image. If the image components and noise are of similar spatial frequencies then at least some noise will necessarily be passed along with the image components. Otherwise, your other choice is to attenuate the noise to a greater degree at the expense of the high frequency components of the image. Simple low pass filtering is not the only option, though. Pattern noise, such as diagonal bands, can be effectively removed in the frequency domain without destroying the high frequency content of the entire image, for example. Also, for spike noise, median filters can be very effective at maintaining high frequency image information (edges) while dramatically reducing the noise. Bruce Girrell <bigirrell@microlinetc.com> 12 Nov 2004

IMAGE ANALYSIS - Cell sizes

I am trying to find information on physical cell sizes, such as diameter (volume) of the nucleus, etc., for various human cell types? Are there on-line resources where this kind of general information is available? Peter Van Osta <pvosta@maia-scientific.com> 20 Oct 2004

Be careful of any data you find. Cells of a given type vary in diameter, and thickness, depending on how they're prepared. Cultured cells spread out very thinly and so will be wider in diameter, and thinner, then, for example, cells collected in preservative, which will "round up" and be smaller in diameter. How much smaller depends on the type and concentration of preservative, and what happens to the cells next (e.g., applied to a slide and fixed, or applied to a slide and allowed to air-dry. Such details should be specified in conjunction with any cell size measurements. For example, there is a 6-fold difference in area between mesothelial cells collected in 50% ethanol that are applied to a slide and wet-fixed, and fresh mesothelial cells that are applied to a slide and air-dried. Gary Gill <garygill@dcla.com> 20 Oct 2004

IMAGING - Digitizing by cameras

This question concerns the way cameras digitize gray levels. I have two cameras in my lab, one is an analog CCD camera (meaning it puts out an NSTC composite video signal), about a dozen years old, and connected to a frame grabber card in a computer; the other is a new digital CCD camera (meaning built in "frame grabber") with acquisition software on the computer. When I look at the image histograms of the same object taken with the two cameras, the histograms are different. In particular, while the older camera generates a more or less smooth curve, the newer one generates a really noisy curve with the number of pixels at adjacent gray levels differing substantially. To put this intuitively, the new camera seems to be noisy in gray-level space. Does anyone know why the two set ups should digitize so differently? It is true that the new camera captures 12-bit images and the old one just 8-bit, but I don't see why having more gray levels should substantially increase the digitization noise. The increase is not small, it really obvious. Tobias Baskin <baskin@bio.umass.edu> 09 Nov 2004

My personal experience is mostly with analog cameras, so my answer is may be not complete. In a CCD camera which provides an analog video signal (NTSC or PAL) the pixel-grid of the camera is resampled into a video signal, where NTSC is 525/60 Lines/Field and PAL is 625/50 Lines/Field. So even if one line of the CCD-grid has more than 525 individual elements, this will be resampled to 525 lines in a NTSC video signal. The discrete pixelation of the image is smoothed into an analog representation. The frame grabber resamples this analog signal back into an digital image with 525 lines (NTSC). An 8-bit frame grabber will resample the voltage range of the video signal into values ranging from 0 to 255. This approach was done to make digital CCD cameras compatible with old analog video systems. This is one component which contributes to what you may see in an image from your analog camera. In the digital camera this back and forth conversion from digital to analog and back to digital does not happen and what you get is the "raw" pixelation at the CCD-grid. If you sample the CCD-pixels at 8- or 12-bit, you express the dynamic range of the CCD into a different subsampling. If you sample a CCD with the same physical characteristics at 12-bit (4096 levels) or 8-bit (256 levels) you will have a finer or coarser readout for the same dynamic range. The 8-bit readout will average adjacent grey levels into 1/128 size steps of the 12-bit system. From what I understand the digital 12-bit image may look "uglier", but better represents what a CCD-grid "sees". Peter Van Osta <pvosta@maia-scientific.com> 10 Nov 2004

EVAPORATION - Evaporating Platinum

A colleague wants to make some Pt mirrors and has asked me to produce them. I am having a great deal of difficulty evaporating the Pt. I have tried tungsten baskets, wires and boats, but when the Pt melts, before it begins to evaporate, the wire or boat breaks. I have tried heating it up slowly and also quickly but with no luck! Can anyone give me any clues as to how I might get the Pt films done? Unfortunately evaporation is the only method I can use. Colin Veitch <colin.veitch@csiro.au> 16 Nov 2004

Have you tried evaporating the Pt alone, i.e. without involving tungsten? Perhaps one or several lengths of 1 mm diameter platinum wire spanning the two boat electrodes might still give off enough metal to make the mirror before melting through. Alternatively, you could remain with tungsten because of its high melting point. The fragility of tungsten may be due to some amalgamation with the Pt, which will also perhaps add to your problem by depositing W also on the mirror. To avoid this, could you perhaps try placing some inert material between the boat and the Pt? I have never tried this, but you might try using a thin bed of sand (or pure silica or even a small piece of coverslip) beneath the Pt. An indirectly heated tiny porcelain crucible with the Pt inside might also work within a tungsten basket. The last and possibly most effective way might be to go to electron beam evaporation (with the gun pointing upwards) because here the substrate and evaporant do not have to bear any mechanical stresses. If you have access to suitable EB electrodes and the associated controller electronics, this would be a logical choice. James Chalcroft <jchalcro@neuro.mpg.de> 16 Nov 2004

The melting point of Pt is well below that of W; 1174 vs. 3410 degrees C and is recommended for evaporation of Pt. The difference in melting points would preclude any W contamination of your mirror. I would use a Tungsten boat rather than a basket because of its robust nature. Maybe the baskets you are using are getting stressed when they are being fixed into the evaporator causing breakage when heated. A Carbon crucible is really required for Pt evaporation by the indirect heating method rather than a ceramic vessel mentioned. James is correct that EB evaporation would give you the best results, not only the quality of the thin film but better control of the final thickness too. However, they are very expensive and you may not have access to them at present. Al Coritz <sampleprep@earthlink.net> 16 Nov 2004

I normally only use platinum for simple shadowing so the quantities may be a bit less than you need for mirrors, but it should work if you're using a reasonably thick tungsten wire filament (0.5 to 1 mm diameter) and reasonably thin platinum wire (0.1 to 0.2 mm diameter) for evaporation.

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I make the filament by slowly bending a V-shape by hand (not too sharp a V perhaps more like a U) then making two more bends to give me the filament shape (___/___). If you bend the tungsten too sharply it tends to greatly weaken it. The other thing to be careful about is that the tungsten wire is not under any tension when it's held in the electrode connectors of the evaporator. If you can't adjust the filament holder then re-bend the wire until it fits. I would then carefully wrap a length of platinum around the pointed tip as tightly as possible. When heating up the filament keep an eye on it through smoked/dark glass and you should be able to see that the platinum is dark as the tungsten glows yellow/white then it will glow and form a droplet at the tip of the tungsten and with very little extra heat it should disappear over a few seconds. If you rush this final stage the platinum can heat unevenly and may even drop off. Even if you're careful you may only get 1 or 2 runs out of a tungsten filament especially if you move or adjust it. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 16 Nov 2004

EDS - quantitative vs. semi-quantitative analysis ?

I would like to understand the difference between quantitative and semi-quantitative EDS analysis. Mike Marks <mikeraj@streamyx.com> 10 Nov 2004

My first response is that "quantitative" numbers come with an error analysis, but hardly any EDS analysis does. In the context of EDS, quantitative would imply "each element measured against its reference standard" and "semi-quantitative" would only imply an attempt to convert X-ray counts to weight percent either by some standardless method, or by at least scaling the spectrum with a minimum of standards (usually one). Michael Shaffer <michael@shaffer.net> 10 Nov 2004

As a minion in a strictly life science EM facility, I probably ought not to express an opinion on EDS analysis - on the other hand, we have been doing it on "life science" specimens in one way or another since 1979, so I can speak to what we used to call "semi-quantitative EDS". There are times when you don't need or simply can't figure out how to get accurate gram atom amount quantitation in specimens (i.e. - virtually any life science sample) but you need to know the relative amount of some element or another and need to have a portable or comparable number to assess a range of specimens. To make a longish story short, we have taken as an internal "standard" an element whose peak area above background remains steady when analyzed under as nearly identical instrument and specimen preparation conditions as possible - say, the calcium level of the thylakoid region of the algal component of a lichen. Knowing that the potassium levels of the same region of the cells is extremely variable when the lichen is exposed to any detectable amount of sulfur derivative stack gasses (or volcano emissions), one can then set up a ratio of the background subtracted potassium peak to the background subtracted calcium peak and then compare the rations across specimens collected from various areas as normalized spectra - say, known distances from a sulfur-containing gas source. You can then build curves that are sort of like dose-response curves and have a baseline to use when assessing the amount of sulfur containing gas that may be/have been present in an area where these lichens grow. Lots of variables, hard to control, certainly not quantitative, but more useful that just looking at randomly collected spectra and guessing - hence, the epithet semi- quantitative EDS. William P. Sharp <wsharp@asu.edu> 10 Nov 2004

'Semi-quantitative analysis' is an expression we have been using for several years to ensure that our students appreciate the limitations of standardless analyses. It is a very useful shorthand expression to distinguish between a full quantitative analysis and a less rigorous standardless analysis. This does not affect the argument that an analysis is either qualitative or quantitative with different degrees of accuracy (or uncertainty) associated with the quantitative analyses. Most of our users accept the limitations of using a semi-quantitative analysis technique instead of making up reference standards, but at least they should understand the limitations, and they can take steps to improve their results. It is too easy for an unaware user to accept the computer's 'quantitative' analysis to several decimal places without questioning it. Ron Doole <ron.doole@materials.oxford.ac.uk> 11 Nov 2004