Thomas E. Phillips
University of Missouri
phillipst@missouri.edu

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

EDS – effect of porosity on measurements

I am interested in is the effect of porosity (or nanoporosity) on EDS measurements. It seems that an increase in porosity should lead to a decrease in the intensity of X-rays, and that this dependence should not be linear. I'll appreciate any lead to a proper publication and/or a short explanation of the problem. Vladimir Dusevich <dusevichv@umkc.edu>

I assume you are talking about area analysis which provides a display of average composition of the scanned area. As you know, dark regions in SEM image are where the e-detector sees less SE/BSE due to a) less generation and/or less survival of them. Reasons for above a) and b) include when the primary beam hits a hole or a pore. Likewise, depending on the size and shape of a pore and the nature of the material, X-rays may not be generated or less may be generated when the primary e-beam has difficulty reaching there or may not survive well (absorbed) in the location of the pore. That is precisely the reason why a reliable quantitative analysis should start with a flat sample surface and why the detector has an important parameter, take-off angle, for quant-routine. X-ray mapping of a homogeneous but uneven sample should provide some insight into this. Chaoying Ni <cni@udel.edu>

A simple way of confirming your proposed relationship of porosity to the EDS values would be to actually measure the porosity of the sample both in terms of total porosity and the size range the porosity occurs in. Nitrogen adsorption is used for porosity less than 30 nm and mercury intrusion for the porosity range of 7 nm to 300 µm. Non-mercury intrusion is used for well characterized materials. Molecular probe techniques are used to characterize size and shape of pores less than 1 nm. J. Roy Nelson <mtl@njcc.com>

The self absorption of X-rays in the specimen is (in most cases) the main process of all influences to generate the X-rays with electron excitation. If the fine focused electron beam is directed to an unknown surface tilt, the absorption effect will be unknown. Quantitative results vary, if there is no estimation of the changed absorption path in specimen. Operators commonly think that if scanning across a larger area of an irregular surface (rough or porous), all regions with more absorption are going to adjust with these regions, which are characteristic for lower absorption. The result should be a spectrum with same absorption (and same results) like the polished specimen of identical element content. But this isn't true! Because of the non linear effects of absorption (e-function), these regions, which have higher absorption, influence the final spectrum more than the others. Because of that, higher absorption always occurs for rough (and porous) surfaces, compared to a flat specimen with same element concentrations. It's possible to prove this fact with mathematics, but not trivial to understand. The opposite is with particles on top of a surface. There it is easier to understand that excited X-rays have lower absorption effects in most cases. Frank Eggert <eggert@mikroanalytik.de>

IMAGE PROCESSING – Pseudo color

In the latest issue of Microscopy Today there is an article on pseudo coloring of images. I was surprised to find that the author says that biolo-

gists nearly always use the "spectrum" color table for the pseudo coloring of grayscale images. I had thought that those who study visual perception had found that among pseudo color scales the "thermal" scale is much better than all the others at providing a good intuitive reading of the image. This is, I think, the scale that Photoshop calls "black body". Can someone please clear up my confusion? Alwyn Eades <jae5@lehigh.edu>

It is unfortunately true that many biologists do use the "spectrum" color scale, largely because it makes "prettier-looking" images. It the cases where they are trying to illustrate quantitative contrast this is not only grossly misleading but it is usually plain wrong and can produce horrific artifacts! The worst offenders are chiefly light microscopists who are trying to represent weak fluorescence contrast and for some reason think it shows up "better" with a spectrum scale. In the STEM/X-Ray/EELS biological microanalysis field most of us use some variation of the "black body" scale which of course more closely parallels the grey scale that is intuitively quantitative anyway (black = 0, shades of grey through white represent more positive values). Relative contrast or non-linear scaling can be achieved by manipulating the scale either continuously or by introducing discontinuities to other scales; of course color then becomes essential (a) because the human eye can perceive considerably more colors than levels of grey, and (b) one can extend the scale over a far greater dynamic range(s); most monitors only display 8-bit levels of grey or 24-bit color but data are often 32-bit or more in dynamic range. The topic of visual perception of data is a fascinating one indeed and has been addressed in several treatises over the years. However in my experience, I have found the most subjectively pleasing results to come from visual artists who seem to have a natural instinct for such representations. A visit to any good art museum should convince most people of this! The "visual perception" of quantitation is a crucial parameter in the use of color images to represent data. It is simply not sufficient, from a perceptual point-of-view, just to have a "text scale" next to some irrational color scale. Of course I agree totally with you that it is easier to distinguish different colors from one another than shades of any color or grey. The question is rather "Is bright green more or less than yellow?" Any quantitative color scale must also have factored in parameters such as Hue, Saturation and Brightness; this is one of the main failings of the "spectrum scale" - it doesn't! Peter Ingram <p.ingram@cellbio.duke.edu>

The most common use of pseudo color is to enhance the contrast of the images and make small details more visible. A little background: Computer monitors are normally set to "True Color". On most graphics cards that means 32 bit of information per pixel, or "millions of colors" as they say. However, each pixel is represented by 3 colors (red, green, and blue), and each of these colors can take on an 8 bit value. 8 bits mean that there are 256 shades of each color available, which can be combined to give you the "millions of colors" (256 x 256 x 256). What is not so obvious, that for gray levels you need to combine the 3 colors in at the same strength, i.e. black is 0,0,0, medium gray is 128,128,128 and white is 255,255,255. This shows, that even if your monitor can display millions of colors, it can usually only show 256 levels of gray. Take into account, that the human eye can distinguish perhaps 50 or so levels of gray and modern cameras can provide anywhere from 4000 to 64,000 levels of gray, and the need for different color schemes becomes obvious. Enter the pseudo colors. There are as many pseudo color schemes as you can think of. Several have become "standards". Among them definitely the "black body" scheme, and the "spectrum" color scheme. The "black body" is perhaps more intuitive, as it basically goes from red to white. This provides a linear scale, which is easy to understand to anybody who has seen a metal heated (and perhaps burned himself or herself), and it is probably easier to discern small contrasts both in the red and the bright parts of the spectrum than in black and white images. However, it does not make full use of the capabilities of a monitor. The "spectrum" pseudo color, on the other hand, makes full use of the available color spectrum, perhaps at the price of an intuitive understanding. It may be better suited to images that have "many" gray levels, which all need to be discerned. If used wrongly, however, the spectrum pseudo color can also lead to misleading coloring. Mike Bode <mb@soft-imaging.com>

In regards to the use and abuse of pseudo color, it is certainly true that human vision can only distinguish a few dozen shades of grey brightness on a display screen, as compared to a few hundred colors. Note that both of these values are far less than the 256 shades of brightness or "millions" of colors that the hardware typically controls. It is also true that trying to direct someone's attention to the "kind of darkish grey spot" is a lot less helpful than "the yellow-orange spot" in an image (but of course, human words for colors aren't terribly consistent or widely agreed, either - look at any set of paint chips in the turquoise-teal-seagreen-etc. family). Pseudo- or false-color certainly has some valid uses. But it is also easily misunderstood, widely abused, and often hides more in the image than it reveals. And if the viewer is one of the 5-10% of men (or the very tiny percentage of women) who has defective color vision, it is inappropriate in any case. Firstly, of course, the table must be shown along with a numerical scale that translates it. But even then, simple spectrum lookup table is rarely if ever a good choice. The problem is that the table is typically constructed with uniform steps in hue, going around the color wheel. But human vision is notably insensitive to changes in hue in the green part of the spectrum, and much more so at the red and blue ends and through the red-to-blue purples. A perceptually uniform hue scale (which I have never seen used) would stretch these out and compress the greens and could probably produce more than a hundred discernible colors. More colors could be seen if they were not fully saturated. Changing saturation and hue in a spiral pattern, or also altering brightness along with hue and saturation, can produce color tables that varied in a gradual way and produced greater ability to distinguish changes. The gradual part is important - if the colors jump around too much in discontinuous ways, the image is badly broken up (camouflaged, in effect) and the overall sense of structure, the gestalt of the image, is hidden. To some extent this happens even with a good, gradual table. The use of the "heat" or "thermal" scale is an example of a gradual and visually attractive scale, which does not break up the content of the image. But it does not actually add very much to the ability to visually distinguish small changes - perhaps a 20-40% improvement over straight grey scale (which is why color tints are also used in photographic printing, to gain the same increase). Note that the brightness increases monotonically in this scale, and that it is by contributing more steps at the dark end that the increase is obtained. For selected purposes, carefully constructed color scales can be useful to help the viewer perceive subtle differences or make comparisons from one part of an image to another. But they need to be documented, and in most cases it is also important to show the original data as well in case the color scale can produce misinterpretation or hide other information. It has been my experience that people are not generally assisted very much by pseudo color scales, as compared to other ways to reveal subtle detail. One of the best of these is to render the surface with elevation representing the original grey scale value. We have millions of years of evolution in our brain wiring that knows how to interpret surface images, in terms of shape and roughness. Using computer graphics to generate properly rendered

images with correct perspective, and adjustable viewpoints, surface characteristics, and illumination is easy with current technology and communicates very effectively. The AFM folks use this trick too, along with color scales, although in most cases in only limited ways. The use and abuse of pseudo color is only one of many issues that have to do with an important topic that underlies just about everything that we as microscopists do - namely, we look at images. But while we are typically very concerned with the performance and specifications of our microscopes, we take for granted the performance of our visual systems, to our peril. Over the past 5 years or so I have been invited several times to give a talk on human vision and how it impacts what microscopists see (and fail to see) in images. At the repeated urging of many people, I've prepared the lecture in written form. Anyone who wants to read it can download the "Seeing the Scientific Image" pdf file from my website (www.DrJohnRuss.com). John Russ <drjohnruss@aol.com>

IMAGE PROCESSING – 12 to 8 bit conversion

I am curious how other software handles 12 to 8 bit conversion of data. Improvision apparently scales directly from 4096 to 255 and 0 to 0 regardless of the image data. It seems to me that you should take the maximum value in 12 bits and scale that to 255 and the minimum value and scale that to 0. Does other software do this differently, or allow you control of the conversion process? I thought that if you needed to highlight low and high values, it would be nice to be able to do this interactively in the conversion process.David Knecht <knecht@uconn.edu>

There are several different issues here, and hence different answers. Autoscaling the image into an 8 bit space will give you the maximum contrast, but it means that each picture will have a different relationship between the original pixel value and the final result, which would make densitometry or anything related to pixel value impossible to calibrate, so absolute conversion by simple division would be preferred in that case. Also, of course, it is much faster, taking only a single pass through the data and that just being a bit shift instead of subtraction and division. Further, there is no good reason to restrict the scaling process to linear. You should perform any gamma correction, equalization, etc. on the full 12 bits before truncating to 8, in order to lose as little of the precision as possible. But why do you want to go to 8 bits anyway? If you have a software package that does not handle 12 bits directly, you would do better to multiply the data up to a 16 bit range and preserve all of the information present. Most modern programs, even the newest version of Photoshop, provide pretty full capabilities for processing and measuring 16 bit per channel images. John Russ <drjohnruss@aol.com>

TEM – magnification calibration

I have been calibrating my recently installed TEM with a grating replica and I need some suggestions. At a print magnification of about 80,000x, I see about a 1% variation in my calculated magnification depending where I select my stop and start marks. How much variation should I expect in magnification due to changes in lens voltage and current? From: <Frank.Karl@degussa.com>

I suspect that these variations are in the grating itself, not your TEM. They can be a bit variable, depending on stretching and buckling from the preparation process. We have a record of measurements of semiconductor standard samples going back 8 years on our TEM, and find reproducibility better than 1% over this time. There is a change in magnification from the centre to the edge of the micrographs on our machine of about 1%, but our microscope is now pretty ancient and I would hope that newer machines are much better than this. Richard Beanland <richard.beanland@bookham.com>

https://doi.org/10.1017/S1551929500052044 Published online by Cambridge University Press

I would recommend the Mag-I-Cal sample for calibrating your TEM. You can do a much larger range of magnification settings than using a replica. I think that the error is 1 to 2%. In addition, you can do rotation and camera constant calibrations with the same sample. One thing to minimize the variation in the microscope is to always change the lenses in the same direction to avoid hysterisis and to make sure that you are at the eucentric point so that the objective lens is the same. Again, you should bring the focus from the same direction. If the goniometer has not been removed or the column split, the values that you get from time to time should be very close. We had to run calibration twice a year on the TEM. I can't remember the range specifically, but I think that the variation in magnification was less than 0.5% and may have been better than 0.1% when the above criteria was met. Scott D. Walck <walck@ppg.com>

If you are using film, a second variation in measurement may come from the enlarger when you print the picture. If the negative is not supported on glass, it can bow in the center and distort the measurement a bit. It was a surprise to me to find that if I had set my "magnification zero" early in the morning and then checked it later in the day, there was frequently a slight change. It was explained to me that in an old building, when there was a greater draw of electricity, a change could be expected and for really important work, I should recalibrate. Am I just gullible? My favorite goof has been the result of not adjusting my tilting specimen holder to read 0 degrees! Pat Connelly <psconnel@sas.upenn.edu>

TEM – Glow discharge

What's the latest word on glow discharge for grids? We have an old vacuum evaporator that will glow discharge, but have hardly ever used it. A new faculty person wants to do more glow discharging and is looking for info. Somewhere I have a paper describing a home made glow discharge device, anybody know about this and if it works? Also have heard that keeping grids in the refrigerator helps too. What's up with that? Her application is carbon films for negatively stained macromolecules. Jonathan Krupp <jmkrupp@cats.ucsc.edu>

The paper describing a home made glow-discharge device is authored by Aebi and Pollard, (1987) J Electron Microsc Tech 7(1): 29-33. We made a similar one (a bit simpler) which we routinely use to charge grids prior to picking up sections and also prior to negative staining. It can probably be made for less than $150.00. I'd be happy to send a jpeg image of the device to anyone who wants it. Doug Keene <drk@shcc.org>

Another way to make coated grids more hydrophilic is to expose them to UV light. I know of one lab that stored Formvar-coated grids on racks under an UV light (did not specify wavelength), and used the oldest ones first. I personally just make grids on a dry day and let them age naturally - probably enough UV here to do the job. But more specifically, a student here at the University of Hawaii tried a lot of different methods of treating her grids, and found that if she put them in their Stratolinker UV Crosslinker (for crosslinking DNA), set it for 30 sec, and pushed the Auto button, it worked great! I looked it up - it uses 254 nm. I don't know how long this effect lasts since they are using their grids immediately. The people who kept their grids on racks under a UV light left the light on all the time, and used the grids soon after taking them out. Another client of ours has used either Bacitracin or protein A to help his viruses stick and to increase wettability of Formvar coated grids with great success. He is no longer here, so I don't have his protocols. When in desperation, I have tried a number of techniques for making coated grids more hydrophilic. The more successful ones include dipping a grid in 70-80% ethanol, shaking off the excess, and then using the grid just as the fluid appears to dry, and I have used a very dilute solution of PhotoFlo, which worked surprisingly well for the application at hand and did not leave a visible residue. I have not yet been desperate enough to try spit! In general, however, I coat a lot of grids on our rare dry day, and then keep them in covered Petri dishes. For Formvar-coated grids, I like them best at about 2 years old, and for carbon films at 6 months or more. I don't know why the become more hydrophilic as they age, and I'm guessing it's some kind of contamination, but I haven't seen anything weird, and they work well for me. This is all to keep from having to repair my vacuum evaporator, of course, but glow discharge is probably the most reliable. Tina Carvalho <tina@pbrc.hawaii.edu>

Usually the reason why one would want to glow discharge treat their carbon coated grids is to make them more hydrophilic. The technique we use is to expose the carbon coated grids to a nitrogen (e.g. air) plasma for roughly 5-10 seconds in an appropriately configured plasma etcher unit. Such a treatment will keep the carbon coatings hydrophilic for roughly 30 days or more. Be aware that not all plasma etchers are appropriate for this purpose. Some etchers operate at 500 watts or higher which will fry the grids so look for one that runs closer to 100 watts. Charles Garber <cgarber@2spi.com>

TEM – Decalcification

We are currently working with a student who is interested in corals and possible virus associations within them. Problems arise when trying to process and section the samples, which contain both normal soft biological tissue and the hard calcified material. Could anyone please suggest a method to decalcify them without doing too much damage to the ultrastructure? Should a decalcification step be done on fresh or fixed tissue? The samples we have to work with now are fixed. Lyn Waterhouse <lyn.waterhouse@adelaide.edu.au>

The best decalcifier to use on biological samples is actually a chelating agent; EDTA ethylene-diaminetetracetic acid. It does not act like a normal acid but binds metallic ions, especially calcium and magnesium. It works better at pH 7-8 and can be used as an aqueous solution or mixed with formaldehyde. It takes longer than the usual decalcifiers such as acids but the results are very good. Dense cortical bone takes about 6-8 weeks to decalcify. If you have x-ray facilities you can monitor the process well. Decalcification must be done on well fixed material; otherwise the decalcifier will macerate the biological matter, particularly the nucleic acids. Evelyn Kaplan <ekaplan@squ.edu.om>

SEM & TEM – same sample

Has anyone ever seen a paper where someone looked at a sample by SEM and then embedded the sample and looked at the exact same cells by TEM? Tom Phillips <phillipst@missouri.edu>

I did quite a bit of this sort of work many years ago. It works quite well although the organelle ultrastructure does suffer a little. Don't expect the same clarity of membranes as a specimen processed directly for TEM. The text 'Principles and Techniques of Scanning Electron Microscopy' by Hayat, has chapters outlining the method. See chapter 5 written by M. Gary Wickham and David M. Worthen and chapter 11 written by Willis K. Paul. Other references at the time: Samuel M. Meller, et al. (1973) Anat Rec 176:245-252 Transmission electron microscopy of critical point dried tissue after observation in the scanning electron microscope. D.E. Scott, et al. (1975) Cell Tissue Research 162:61-73 The primate median eminence. Correlative scanning transmission electron microscopy. D.E. Scott, et al. (1978) Cell Tissue Research 190:317-336 Correlative scanning-transmission

NETNOTES

electron microscopic examination of the perinatal rat brain. W.R. Gillet et al (1991) Human Reproduction 6:645-660 and W.R. Gillet et al (1992) Human Reproduction 7:446 – 452 both deal with human ovary, examined in SEM then same sample examined in TEM. Allan Mitchell <allan.mitchell@stonebow.otago.ac.nz>

I had a paper sometime ago where we did just this. The paper is in Microscopy Research and Technique (1995) 31:174. We developed this method to look at low level contaminants in soils. Edgar C. Buck <edgar.buck@pnl.gov>

I did this with horse uterus. I processed as for TEM with good fixation and osmium, critical point dried, examined with SEM, and then went back into alcohol for several hours and run-up into resin. I was very surprised at the quality of the results we got, though morphological preservation was certainly not as good as straight TEM. There were holes in the matrix presumably from extraction and precipitation of things during the drying, but cellular structures were recognizable. We were after ciliary structure and they were well preserved. The metal coating did not interfere with anything but provided good evidence of how well (or not well) the surface was coated. Slightly distracting in the photos so maybe carbon coating would be better. Scott Whittaker <whittaker.scott@nmnh.si.edu>

I have done this in the past working with very small delicate plant specimens as well as medical specimens of human ear tissue. We published a paper about the holder we designed to process the gametophyte from flowering plants. This holder was developed to critical point dry the embryo sacs for stereo TEM and then later further processed for conventional TEM. Our principal interests were to make stereo images of the specimens in a 200kv TEM so that we could see cytoskeletal and structural organization. Basically we wanted whole mounts so we could investigate the 3-D organization of these cells and then augment it with conventional TEM images. They would have certainly have been perfectly good SEM samples had we found it necessary to look at them that way. A micro-sample critical point drying device for small SEM and TEM specimens. 1990. G.W. Strout, S.D. Russell. J. Elec. Micros. Tech. 14:175-176. The human ear tissue was something that was primarily to be looked at in the SEM and then secondarily processed for TEM. I would point out that this was the intent from the very beginning and so the processing (fixation etc.) was from a TEM perspective from the very start. I do not know if this work has been published. In both cases we were very fastidious about critical point drying the samples. We used multiple exchanges of liquid carbon dioxide by filling, soaking, flushing then repeating many times. Greg Strout <gstrout@ou.edu>