# NetNotes

**Edited by Thomas E. Phillips**
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Selected postings from the Microscopy Listserver from May 1, 2015 to June 30, 2015. 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:
### glycogen

*For several years, we have lost the ability to visualize glycogen by thin section TEM. As I recall, this occurred when we changed bottles of DBP in our Epon-substitute/Araldite mixture. Has anyone else had the glycogen "disappear", and come up with a solution?* ***Cynthia Goldsmith cgoldsmith@cdc.gov Tue May 12***

Glycogen can become coalesced into amorphous unstained regions in the cytoplasm under certain fixing/staining conditions. I have found this in certain cell types (i.e., muscle, lung tissue) if you use non-reduced osmium followed by un-buffered uranyl acetate (aqueous) *en bloc* staining. Generally if you use non-reduced osmium, I would *en bloc* with buffered (0.1 M maleate, pH 6.2) 2% uranyl acetate. If you do use reduced osmium ($K_4Fe(CN)_6$), then you can continue with aqueous uranyl acetate. A remedy for material already in plastic is to triple stain the sections: 1% filtered tannic acid (10 min) followed by 2% aqueous uranyl acetate, then lead citrate. This will fill in the amorphous regions and stain the glycogen there. I have done this with lung tissue. Michael Delannoy mdelann1@jhmi.edu Wed May 13

## Specimen Preparation:
### mitochondrial contrast

*We have a few mammalian skeletal muscle samples that are already embedded in Spurr's resin. They were conventionally fixed with glutaraldehyde and $OsO_4$, and en bloc stained with uranyl acetate. The mitochondria are poorly contrasted within the muscle fibers using uranyl acetate (50% ethanolic solution) and lead citrate post-staining. Section thickness is 70 nm. Are there any modified post-staining techniques that can preferentially enhance contrast of the mitochondria vs the surrounding muscle fibers?* ***David Lowry dlowry@asu.edu Tue May 26***

You can try triple staining with 1% filtered and aqueous tannic acid (10 min) before the uranyl acetate and lead. The grids must be Formvar coated as the copper will react with the tannic acid. Michael Delannoy mdelann1@jhmi.edu Wed May 27

## Specimen Preparation:
### non-radioactive uranyl acetate

*I just read a post on uranyl acetate and wanted to ask you a question regarding a particular uranyl acetate sold by EMS as non-radioactive. Does this work the same as radioactive ones? Has anybody used this non-radioactive uranyl acetate? Does it have any kind of limitations? Their description is at this website:* http://www.emsdiasum.com/microscopy/products/chemicals/tannic.aspx#22400 ***Fumiya Watanabe fxwatanabe@ualr.edu Thu May 21***

Uranyl acetate is always radioactive. There are uranium isotopes whose decay rates are quite low, and in some states, the total radioactivity is deemed low enough so that uranyl acetate can be discarded down the sink. I expect that this is what EMS has in mind. Bill Tivol wtivol@sbcglobal.net Fri May 22

As mentioned by Bill, uranyl acetate is at best depleted, but never non-radioactive. What you came across is actually a non-radioactive uranyl acetate substitute, which consist of "Two lanthanide salts, samarium and gadolinium triacetate". Check the paper quoted in the data sheet. As a general note, the staining properties of uranyl acetate vary substantially between manufacturers and thus between countries (as nobody wants to ship the stuff unless necessary). Testing is the only way to find out. Chris Buser cbuser125@gmail.com Fri May 22

## Image Analysis:
### particle sizing

*I had an animated discussion with a colleague material scientist. The question is: is it possible to measure the size of microparticles (some aluminum-containing mineral) by embedding them, preparing a fine flat surface, coating and analyzing in REM with W gun in BSE mode. My colleague says it is no problem, really. I say I see 2 objections (really): - the first one is that the interaction volume is much bigger in BSE mode than in SE mode (Mr. Chapman's rule #3), decreasing the lateral resolution, which is already a problem for particle sizing in REM - the second one is more theoretical: by sectioning microparticles, we get all sorts of cross-sections. By measuring the diameter of the cross-sections we don't get the measure of the mean diameter of the particles but a much more spread size distribution which is not centered on the actual diameter of the particles.* ***Stephane Nizets nizets2@yahoo.com Tue Jun 2***

You are right. The problem of size distribution caused by serial sectioning can be overcame by a) Assuming all of the particles are the same size and are randomly distributed in the embedding medium - dangerous assumptions at best or b) Serial sectioning the block and imaging the block face - this requires an ultramicrotome within the SEM (= REM) chamber, but such things exist now: Leighton, S.B. 1981. SEM images of block faces, cut by a miniature microtome within the SEM - a technical note. *Scan Electron Microsc* 1981, (Pt 2): 73–6. Wanner, A.A., et al., 2015. "Challenges of microtome-based serial block-face scanning electron microscopy in neuroscience." *J Microscopy* doi: 10.1111/jmi.12244 or c) Micro-CT within the SEM or by itself. This still leaves all of the issues in your first objection and the basic "where's the edge?" question. Even in secondary imaging this is an issue. How important depends on the particle size - if they're micrometers in size the error won't be too big relative to the particle; if they're nanometers in size, the error can be relatively big. Either way, why not use light or TEM microscopy to size the particles? These would be more accurate. Phil Oshel oshel1pe@cmich.edu Tue Jun 2

Interaction volume in BSE is bigger than in SE but is not so big if accelerating voltage is low enough. This volume can be small enough, at low voltage, if the mean atomic number of particles is not too poor. Many BSE detectors are now efficient under 5kV; if you

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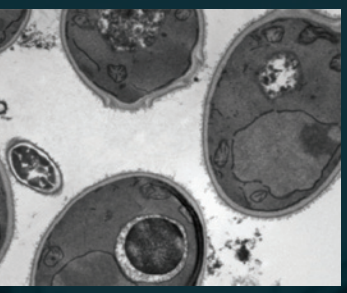

*Yeast cells were fixed with glutaraldehyde in cacodylate buffer, washed in distilled water and postfixed with 1% KMnO4 in distilled water*

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# EMS has it!

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can use a FE SEM with a good BSE detector just above the sample (some millimeters) you may obtain a good result. More than lateral resolution, the precision of the measure may be affected by the calibration of magnification. For such job it could be interesting to check the accuracy of the magnification with an appropriate standard. You can easily simulate how far is the interaction volume for your sample by using software like CASINO for example. If you are lucky, you may find a good value for acceleration voltage; not too deep on the sample to avoid resolution problem but deep enough to get BSE from all the volume of the particles. In that case, probably you will see the diameter of the particle and not only the diameter of the sectioned disk on the surface. Of course, it supposes good correlation between several factors: Z number of particles, Z number of matrix, size of particles, etc. Nicolas Stephant nicolas.stephant@univ-nantes.fr Tue Jun 2

Why do you like a complicated approach to a simple question? Remove the bulk substrate! The easiest would be to cast a water or ethanol suspension of your particles on a TEM C-film/Cu 200 mesh grid, then go to see a colleague who runs a TEM or STEM microscope! Too far? Too expensive? No friend? Be self-standing. 1. First obvious solution: stick your TEM grid on top of a 10 mm deep, 2 mm diameter hole drilled in any light conductive material block (Al alloy is fine). Look at it in SE mode as usual, you will avoid all the trouble of electrons spreading in the substrate, BSE backscattering and secondaries of type 2. Of course, the W cathode is not as good as a FEG for ultimate resolution, but nevertheless will give a better resolution than BSE. 2. Assuming you have a semi-conductor BSE detector, you can easily transform your SEM in S(T)EM microscope. With a bit of manual skill you can carefully mount your BSE detector on the sample table (upside-down to receive the primary probe!), then with a light U-shape jig hold the TEM grid some 10-15 mm above the center of the BSE detector. The BSE signal becomes a STEM one. However the contrast is not well defined as both directly transmitted (bright field) and scattered electrons (dark field (DF) + high-angle annular dark field (HAADF)) are detected together. 3. To improve the contrast, you may add a beam stop to catch the transmitted beam and let only the scattered electrons to reach the BSE detector (DF + HAADF). Or you may cover the BSE detector, letting only a 2 mm hole to get the directly transmitted beam and BF contrast. Lazy? You may even more easily mount the BSE detector off-center to block the directly transmitted electrons on its edge and detect only electrons scattered in 2 quadrants (DF + HAADF, 50% signal). The efficiency of detection is high, which means you may use lower beam currents than usual and certainly much lower than for BSE. Expect a better resolution in this STEM mode than in SE. Beware! Contamination may be a limiting factor depending on your vacuum quality and require you to work fast, even to record pictures on fresh areas adjacent to that used for focusing. Philippe Buffat philippe.buffat@epfl.ch Tue Jun 2

## Image Processing:

### convert to .dm3 files

*Digital Micrograph records EEL spectra as .dm3 files. While there are scripts that can export the data to a text file - I am wondering whether the reverse is possible. I have a two column.txt file of my EELS data - will it be possible to import that data into Digital Micrograph for further processing?* ***Debangshu Mukherjee debangshu@psu.edu Wed Jun 3***

There is a feature in the GMS "File" menu called "Import Data...". This is a fully functioned import tool that handles most data types. For importing EELS data, the spectra must be represented in equally spaced energy bins. If it is just a single row of data with the intensity values, you can convert this to EELS and calibrate using the "Spectrum" menu items. If the data is in energy / intensity format, there is no simple way to convert it to EELS data. I have written a short DMScript that handles imported data where the first row (or column) contains the energy values and the second row (or column) contains the EELS counts. I hope this helps, Ray Disclosure: I work for Gatan but I am not a programmer. I am sure the code below breaks a dozen rules. I learned scripting as a user of Gatan equipment and continue to use it when analyzing data, trying out new ideas or prototyping. This is unsupported script code. Interested readers can e-mail me to receive copy of the code. Ray D. Twesten ray.twesten@sbcglobal.net Wed Jun 3

## Image Processing:

### Photoshop

*We've updated our free image processing instructions from the NUANCE Center at Northwestern University. A few additions have been made to include explanations of file types, how to resize images the right way, and even some new coloring instructions. All of which are designed to be super easy and will walk the user step-by-(sometimes painful) step to do all sorts of normal image processing procedures, to several different procedures to apply false color to an image. The extra special part is the chapter on what I call Multi-Detector Color. Now I know I did not invent this technique, but I've worked out some pretty simple procedures that will allow you to make these really fantastic color images, even if you only have 1 SE detector in your SEM. It's totally free, so please check it out and let us know what you think.* http://www.nuance.northwestern.edu/docs/epic-pdf/Basic_Photoshop_for_Electron_Microscopy_06-2015.pdf ***Eric Jay Miller eric-miller@northwestern.edu Tue Jun 9***

## EM:

### Gauss meter

*I was thinking of buying a portable Gauss meter last week to try and track down some electrical interference in our lab. Comment from a colleague – 'oh, just download a free app for your phone'. So I did; I think there are several ones available but I got Teslameter. It looks really good - and seems to work with reading levels down to 0.1 mG. Does anyone know if these apps actually work as well they appear to? Are they accurate?* ***Richard Beanland contact@integrityscientific.com Fri May 22***

There are I think two dimensions to this question. The first is technical. If you are trying to track down sources of interference in your lab, you are probably concerned with time-varying magnetic fields - often at 60 Hz. The sensors in a phone will give DC readings, and thus they are not useful to you. I bought a Tenmars TM-192 3-axis EMF meter (<$200), which I compared to a $2000 Hall effect probe, and I found it to compare nicely for purposes of walking around a space and seeing where "hot spots" are. The second dimension is economic. Do you want to base expensive decisions such as room design, instrument selection, instrument placement or interference abatement on an app you got for free? Be sure your investment in information gathering is in proportion to the commitments you make based on the information. Larry Scipioni les@zsgenetics.com Fri May 22

## EM:

### high dynamic range

*I was wondering if anyone has ever come up with a good repeatable technique for making high dynamic range (HDR) images using SEM or TEM micrographs. I think this could be a useful technique to use on low contrast samples and just to make a cool image. But I can't seem to get it to work on my own.* ***Eric Jay Miller eric-miller@northwestern.edu Wed Jun 17***

https://doi.org/10.1017/S1551929515000735 Published online by Cambridge University Press

There are two issues, it seems: pixel depth and look-up table. I was a little confused by your statement of low contrast images, for HDR is usually used for high contrast images, where some places are saturated and/or some are lost in the black. That issue beside, with an 8-bit detector signal, one could take two images - at lower and higher gain - and then map the two onto a single 16 or 32 bit image. Then the gray levels need to be compressed into a non-linear scale that allows the wide range to show in a human-detectable fashion. A method used in astronomy is the wavelet-log representation, for example, where the pixel values are converted to a log scale. This allows many orders of magnitude of signal to be represented in a nice way visually. See Starck and Bobin, *Astronomical Data Analysis*, 2009, Equation (2). Of course, you must start with a high bit-depth image. I wrote an ImageJ macro that does such a wavelet transform, and my meager testing found it to be good for bringing out faint details in 32-bit STEM images. Low contrast images don't look good, for they get compressed into too few gray levels. I would be glad to send it to you offline, as the listserver doesn't digest attachments. Larry Scipioni les@zsgenetics.com Thu Jun 18

We did this for TEM some time ago and finally got round to publishing a little letter on it last year: see *Microscopy and Microanalysis* 20 1601-4 (2014), DOI: 10.1017/S1431927614012975. Also at https://www.researchgate.net/publication/266246078_High_dynamic_range_electron_imaging_the_new_standard. You should be able to find the Digital Micrograph script there too, under supplementary resources. It doesn't help with low contrast samples, but it does help with very high contrast data (e.g. diffraction patterns). For low contrast samples it would be better to use a phase plate ($$$) or a through-focal series to produce an exit wave reconstruction (or in-line holography, however you want to label it). Richard Beanland contact@integrityscientific.com Thu Jun 18

Thank you all for the suggestions. I confused myself and misspoke when I said low contrast images. Usually when you have an image with very bright or very dark areas in it, you'll need to take a very low contrast image to make all of the data visible. I just think this is an interesting technique for data as well as artistic reasons that should be able to be applied to EM. Just haven't found an easy way of making it happen yet. Eric Miller eric-miller@northwestern.edu Thu Jun 18

## TEM:

### pressurized air vs. nitrogen gas

*Yet again a new chapter in the life of a biologist responsible for a FEI Tecnai G20! Our TEM is connected to a $N_2$ cylinder to deliver the pressure necessary for the valves to work. Now we got a new air compressor with all the necessary filters to avoid having water in the lines so I want to use it to feed the TEM valves with pressurized air. In the water/air rack, we have 2 manometers. The first one limits the pressure to 5 bar, the lines are labeled CCD camera, blow off (whatever it is) and probably the cushioning of the microscope (it is labeled "S34"). The second one is connected to the first one and limits the pressure to 0.3 bar, it is used for the TEM Valves. I am concerned with the use of pressurized air in state of $N_2$ because the second manometer has a label "$N_2$ gas". So my questions: Can I safely connect pressurized air in place of $N_2$? - Is there any reason why I would absolutely need a $N_2$ line? (e.g., for flushing during the procedure of $SF_6$ filling, we bring a cylinder next to the microscope).* ***Stephane Nizets nizets2@yahoo.com Tue May 19***

You can use pressurized air as your main air supply. The $N_2$ line is for back filling as in a filament exchange, column vent or camera vent. Bringing in a gas cylinder when needed works well. John Schreiber js51@princeton.edu Tue May 19

## SEM:

### proximity of sputter coater to SEM

*I'm in the process of reorganizing my lab and I currently have my sputter coater and JEOL JCM-6000 Benchtop SEM placed 115 cm apart. My concern is that if the column is open to the atmosphere and the sputter coater is run that the column may get contaminated. Is this a legitimate concern?* ***Lisa Stafford lstafford@sono-tek.com Wed May 6***

With all due respect I think you have your priorities wrong! A sputter coater, like any other piece of equipment using a rotary pump, can be a contamination device due to the pump fluid vapor which is brought out of the pump with the gas. This contamination will pollute the laboratory air, fall on surfaces and on people. But even these problems should not worry you as much as your staff breathing this contaminated air! Please make sure you use the best possible filter on the pump, and change it regularly; to wait until you smell the contamination, in my mind, too late! Alternatively, vent the pump to the outside world. Have in mind that the way to clean out some of the contamination within any rotary pump fluid is to run a high level of air/gas through it. As the sputter coater rotary pump starts to operate it ejects a high level of gas which will contain pump fluid. Even under its actual operating conditions you are purging gas into the system that is also passing through the pump fluid. Add to this that the sputter coater is usually the first instrument to put the specimens under vacuum, thus it has all the vapors from the specimen to cope with. This combination of vapors and chemical reactions within the pump fluid, result in the smell you sense if the filter fitted to the pump is unable to cope. I do hope that this helps you and the many others who should be aware of rotary pump vapors? Steve Chapman protrain@emcourses.com Thu May 7

## STEM:

### polymer blends

*What is the best imaging mode\setup to image polymer blends samples? Actually, I am trying to image a sample that contains PTB7:PC71BM with DIO. Basically what I am looking for is to generate some contrast between these three materials. The first one is a sulfur-rich while the second rich with carbon. DIO is Iodine rich. We tried to use HAADF and dark field imaging in STEM with low convergence angle probe in the objective off mode. Unfortunately, it was not successful. We are not keen to attempt anything with EFTEM, as the doses required to get good signal to noise are rather high and likely to fry the sample. Additionally, finding small amounts of I using EFTEM will be very hard (slow rising edge at a fairly high energy of 619 eV gives very weak signal and therefore requires a huge dose to see it, which probably destroys the sample).* ***Ala' Afeef alaa.afeef@gmail.com Wed May 13***

If all you want is contrast then plasmonic imaging in EELS will be much, much lower dose than core loss EELS. Here's an example of plasmonic EELS imaging of block copolymers which will give you some idea of what can be done this way. http://www.sciencedirect.com/science/article/pii/S0304399110003256# Zack Gainsforth zackg@berkeley.edu Wed May 13

## STEM-EDS:

### software quantification

*I've been trying to decipher the quantification options for our STEM-EDS analysis program. The program is called "Analysis Station" and its sub-program is "MappingProgram." It gives me three options: * ZAF * Ratio * NET Int. I have figured out what ZAF is, but I'm not sure about the other two. Does anyone know if these routines automatically deconvolve overlapping peaks (judging by the appearance of my maps they do…)? Moreover, does anyone happen to have a manual*

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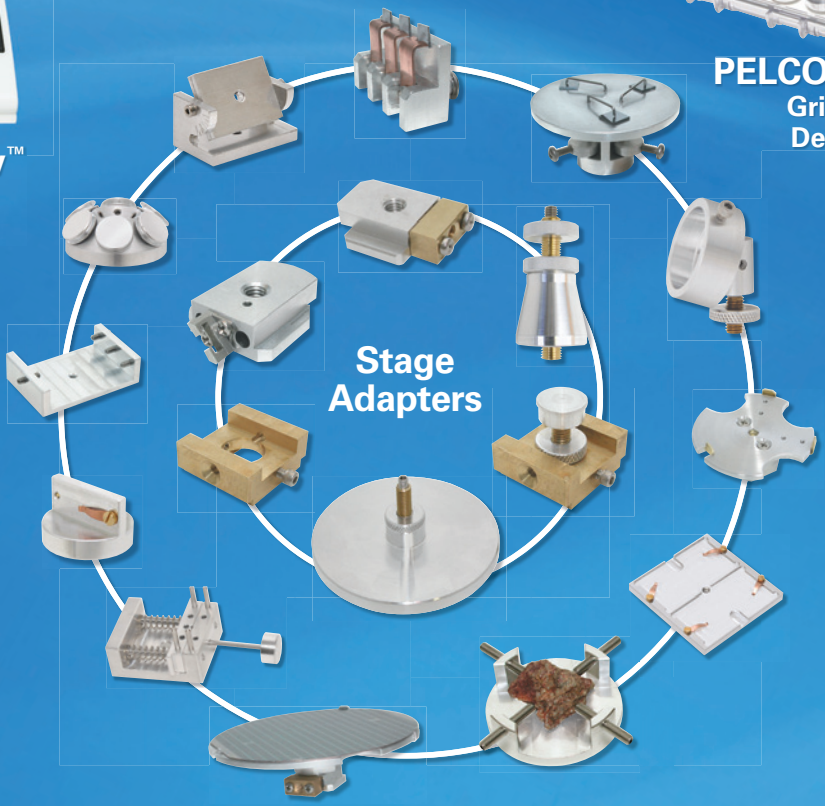


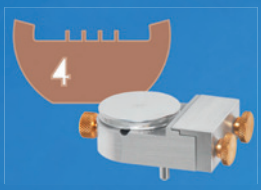

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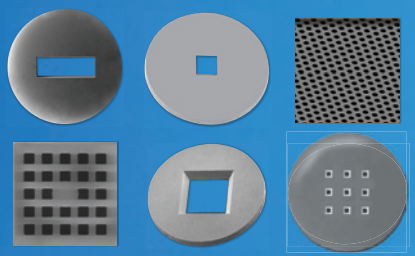

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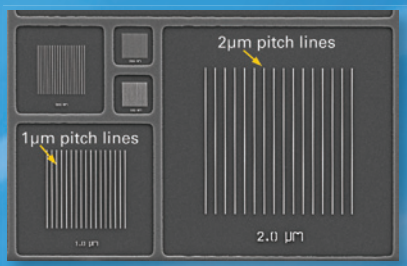


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*that describes what the various options are for each routine?* ***Steven R. Spurgeon steven.spurgeon@pnnl.gov Mon Jun 15***

The JEOL EDS software works on both SEMs and TEMs and the three different quantification methods are tailored to those two different microscopy techniques. In short, the quantification routines are used to compute the k-factors for quantitative analysis. The ZAF routine is a method to compute matrix corrections for the effects of atomic number, likelihood of absorption, and characteristic fluorescence of emitted x-rays from your sample, and these corrections are generally only valid when you have the classic teardrop interaction volume (i.e. bulk samples). So this would be a good routine to correct maps acquired via SEM. The ratio technique is the appropriate technique for maps acquired via TEM as TEM samples are typically much too thin for the emitted x-rays to be influenced in any statistically significant manner by Z, A, or F (you might still need to worry about channeling effects induced by sample orientation, however). This routine uses precomputed k-factors or experimentally calculated k-factors as anchor points from which the quantitative composition of your sample is derived, as the ratio of peak intensities from a known material and an unknown material is directly related to the k-factor. Selecting the ratio routine fills in the peak intensity of a known material and the k-factor, allowing the software to calculate the concentration from the measured peak intensities of your unknown sample. Obviously your results will be much more quantitative should you experimentally calculate k-factors for the elements of interest (and under identical illumination conditions) than by using the software database. X-from the JEOL EDS software manual regarding the net intensity routine: "The net intensity map displays relative intensities after deconvolution and back-ground subtraction. The result of the net intensity map is different between with check in the "100%" and without. Check "100%" Concentration before correcting by Quant. Method displays. The summation of K-ratios for all elements is unified at each pixel. Uncheck "100%" Normalize the maximum net intensity to 255 in the all pixel of the element." Practically speaking, you'll want to be exceedingly careful generating quantitative EDS data without first experimentally generating appropriate k-factors. Without doing so, your quantitative numbers are going to be ballpark estimates for anything but the simplest samples. Such data might not be trustworthy by itself but it can be useful as a comparison between EDS data from different unknowns acquired under similar conditions. Masashi Watanabe and David Williams discuss the problems associated with acquisition of quantitative EDS data and an alternative to using k-factors in a series of papers (see M. Watanabe's website at Lehigh) and in the second edition of the classic Williams and Carter intro to TEM text. Chris Winkler microwink@gmail.com Mon Jun 15

## STEM-EDS:
### beam broadening

*I am looking for a program that will help me calculate beam broadening for STEM-EDS measurements. In particular, I'd like to figure out the maximum achievable spatial resolution given a known sample composition, zone axis, thickness, energy, and so on. Does anyone know if there is a freely available program that can help me with these calculations?* ***Steven R. Spurgeon steven.spurgeon@pnnl.gov Thu Jun 11***

David Joy wrote such a program some time ago. I hope someone else on the list knows how to get a hold of it. Bill Tivol wtivol@sbcglobal.net Thu Jun 11

You can also try using the Monte Carlo programs by Gauvin and Demers group (WinXray and Casino) or DTSA-II (N. Ritchie, NIST). The cross-sections are more accurate for SEM work, but they should be good enough to give you an idea of your beam spreading. DTSA-II has an option for a thin film on substrate and you can define the substrate as "none". Win X-ray MC X-ray http://montecarlomodeling.mcgill.ca/software/softwareprojects.html Casino http://www.gel.usherbrooke.ca/casino/ DTSA-II http://www.cstl.nist.gov/div837/837.02/epq/dtsa2/ Hendrik O. Colijn colijn.1@osu.edu Mon Jun 15

I am one of the authors of some of the programs mentioned, and I want to add a warning. All MC programs mentioned do not take into account the crystal structure of the sample, they consider the sample amorphous. So you will get the beam broadening without channeling effect (or orientation effect). For more information, we published a paper on the lateral resolution in STEM mode with CASINO v3 in amorphous sample: Demers, H.; Ramachandra, R.; Drouin, D. & de Jonge, N., "The Probe Profile and Lateral Resolution of Scanning Transmission Electron Microscopy of Thick Specimens," *Microscopy and Microanalysis*, 2012, 18:582-590 DOI: 10.1017/S1431927612000232 Hendrix Demers drix00@gmail.com Mon Jun 15

Good point! All the MC programs I mentioned assume an amorphous sample. I had missed the fact that Steve had included channeling in his requirements. I believe that Dave Muller's group at Cornell has taken channeling into account in their atomic resolution STEM calculations. For non-atomic resolution work, channeling may safely be neglected in most cases. It is also likely that beam convergence angle will have a more significant effect on resolution than channeling. For example, in organic samples <100 nm, Muller's group indicate that the beam convergence has a greater effect than the beam spreading (Ultra. v.109 p.1 2008). Hendrik O. Colijn colijn.1@osu.edu Mon Jun 15

MT

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