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NETNOTES

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Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 10/10/05 to 12/12/05. Postings may have been edited to conserve space or for clarity.

LM - polarization microscopy with Nicol prisms

I am doing a research project on William Nicol who invented the polarizing microscope, but I can't seem to find any biographical information on him. Is there anyway you can help me? Irene Zepeda <familia_zepeda@yahoo.com> 21 Oct 2005

William Nicol invented the 'Nicol prism' in 1828. This polarizer prism is "a rhombohedren of calcite, cut diagonally, ground and polished and cemented together with Canada balsam". However not many living microscopists will have ever used one, as Edwin Land subsequently invented 'Polaroid' plastic sheet in 1932 and this is now always used in place of the Nicol prism as the polarizer/analyzer. Keith J Morris <keith.morris@ucl.ac.uk> 21 Oct 2005

My first polarizing scope had a Nicol polarizer and cap analyzer. I'm still using that scope as the balsam hasn't separated. Oh, yes it has a mirror and I use a focusable lamp. I used to do dispersion staining for asbestos and microchemical tests with that scope. Thinking of it brings back memories. Frank Karl <frank.karl@degussa.com> 21 Oct 2005

I think that the Nicol type prism provides high quality extinction, better than the Land type. I expect there have been modern refinements to the design since Nicol, but a pair of cemented appropriately cut crystals provide excellent extinction. I believe they are common on optical benches in physics applications -- it can be awkward working the prisms into a microscope light path. Tobias Baskin <baskin@bio.umass.edu> 21 Oct 2005

Another problem with the plastic polarizing filters is that they fade quickly or melt outright under intense light sources, e.g. a Xenon lamp. I wonder if the cement used in the cut crystal prisms would also experience a problem with high light intensity. I found that heat filters did not help; neutral density filters did help but defeated the purpose of the high intensity lamp we were trying to use. Damian Neuberger <neuberger1234@comcast.net> 21 Oct 2005

LM - reflecting objectives

While rooting around in a cupboard this morning, I came across a couple of Beck "reflecting objectives" in their little mahogany boxes. I would dearly like to know what they are used for - it's clear that they focus parallel light to a point. Do they have a use in microscopy, or have I just found a component from a rather old experiment on an optical bench? Richard Beanland <richard.beanland@bookham.com> 07 Nov 2005

Reflective objectives had several different proposes. One was an attempt to create better images as compared to the crude glass lens of the late 1800 - early 1900's. They also had a purpose as they would gather and focus IR and UV. Of course you needed to use film to collect and "see" the image. I seem to remember that later ones sometimes contained a glass lens to assist in correcting for visible light images. Of course you lost all the UV/IR that the glass filtered. Modern IR collecting objectives used in micro spectroscopy are reflective objectives revisited and we have come full circle. Frank Karl <frank.karl@degussa.com> 07 Nov 2005

Another useful aspect of reflective objectives is that they can maintain a large NA at a long working distance and hence get better resolution than glass lenses at similar long working distance. Kind of like a telescope. John Mardinly <john.mardinly@intel.com> 07 Nov 2005

They are almost certainly microscope objectives. Put one on a scope and see how it works. The principle behind them is mirrors have almost the same focal length over a very wide range of frequencies. The small exception is explained on this Canon page on Near Field Microscopy http://www.canon.com/technology/s_labo/light/004/01.html. In the Dripping Light selection, it shows that a reflected wave leaves the surface 1 wave length away from where it strikes the surface. But for most practical uses that does not affect us. If they are low power and low NA, the best way to use them for near infrared and the UV that silicone sensors will capture is to see if they will work as a prime lens for a video camera with no lenses to have different focal points at 1000 nm light and 300 nm light. The lens will cover a much wider range of light than silicon will detect, but you start taking about a great deal of money for cameras. You also need a monochromator or filters to make much sense of the images. Gordon Couger <gcc@couger.com> 07 Nov 2005

LM - fading sections

We have been using a 1% toluidine blue O in 1% sodium borate as a general stain for all of our thick sections (1-2 microns). We use Cytoseal 60 as the mounting medium. We have noticed that the sections are fading more quickly than they have in the past and sometimes we get "round clear droplets" on the sections. Can anyone recommend a stain that won't fade so quickly? Does anyone have a clue as to the nature of the "droplets"? Stacey Andringa <stacey.andringa@uc.edu> 09 Nov 2005

The problem with fading stains (usually highly alkaline solutions of basic dyes like toluidine blue in borax solution) on plastic resin semi-thin sections for me is a long known fact (regardless of the mounting media I used earlier).....so I started some 20 years ago to change my processing and archiving mode for semi-thin sections. We have to store the semi-thins of our cases for legal reasons, if possible, up to 20 years. i) For looking at stained sections for orientation purposes, most of them also for diagnosing, as well as trimming the right location for EM-ultrathin sections we do not mount them with a slide and/or mounting medium. We find the spatial resolution already achieved without mounting a coverslip (compared to a thick histological, paraffin-embedded section) is enough to choose the location of ultrathin section trimming. ii) For photographic documentation or high resolution light microscopy of selected stained sections, we immerse the respective sections with a small drop of immersion oil, view either with a coverslip 0.17 mm placed carefully on the oil drop. After having done this, the coverslip will be slipped away, the object slide will be immersed in (a) Coplin jar(s) filled with xylol (2 x 30 sec. each) or any other diluent which will dissolve the immersion oil. Afterwards we give the object slides a blast of compressed air by means of a nozzle connected by a tube to a bottle or the pipeline of compressed air and the mounted sections are ready for storing/archiving. If you do that in a light protected, dust-free place, you will not see - at least over 5 years from my experience - a fading of staining, also you will not

have bad wrinkles in your sections which are produced over time due to the mounting agent. This creates the second advantage, the first being economizing the mounting process (in avoiding permanent mounting with not very healthy mounting media as well as saving costs for coverslips and the time needed for coverslipping, drying of mounts, etc.) If in case the staining of sections does not display brilliantly after a long period of time as you are expecting, you are able to re-stain your sections easily and rapidly with the routine staining procedure without loss of substantial information. Wolfgang Muss <w.muss@salk.at> 9 Nov 2005

The droplets are likely water from incomplete dehydration and clearing. Cytoseal is a toluene based mounting medium that will not mix with water. Extend your dehydration times and/or add an additional absolute ethanol step, and maybe add another step in clearing agent. Make sure you replace the dehydration baths and clearing baths on a regular basis as water will gradually build up in them. Although a greater issue with thicker paraffin or Vibratome sections, it can occur with plastic sections if they are rushed through or the baths have not been changed. What is the time frame of your fading? Days, months or years? Çould it be the pH of the Cytoseal? We get a few years with Toluidine Blue mounted in DPX. Glen MacDonald <glenmac@u.washington.edu> 09 Nov 2005

After discovering that many of our older toluidine blue stained semi-thin sections that had been mounted with a commercial mounting medium had faded, I decided to try using our standard Epon-Araldite-DDSA embedding resin as a mounting medium. Simply put a drop or two of resin (we use left over resin from embeddings, stored in vials in a freezer) on the dry, stained slides, and coverslip slowly to avoid bubbles. The slides are viewable immediately, and will harden in a few days on their own, or overnight at 60 degrees. They should not be overheated or some destaining or wrinkling may occur. Since switching to Epon as a mounting medium, I have not seen any problem with fading. As for viewing slides before permanent mounting, the image can be greatly improved just by placing a coverslip over the dry section while viewing. Most objectives are corrected for the presence of a coverslip, so even with nothing but air between the coverslip and the section, the image is very good. Ralph Common <rcommon@msu.edu> 16 Nov 2005

COLLOIDAL GOLD — cryo-thin section labeling

I have a question for all those that have done immunogold labeling on cryo-thin sections. I am having trouble getting consistent, predictable results. My signal to noise ratio seems variable which makes it difficult to standardize the protocol. One thing that I have noticed is a 5-15x increase in signal (based on gold count) if I use just BSA and omit any normal serum. I also see an increase in background but in seemingly random patterns. Sometimes I think the positives are much cleaner than the minus primary controls. The protocol that I am using at present is: place freshly picked up sections on droplet of 0.05M glycine / 1% BSA in TBS (1 hr), then 1% BSA in TBS (1 hr), primary antibody overnight (1% BSA/TBS), secondary (ultrasmall up to 20 nm gold) for (2 hr), fix, embed and view. My previous standard protocol was including 5-10% normal serum in all the above solutions. Has anyone else seen this increase in labeling frequency? I would be interested in protocols that people are very pleased with and give consistent clean results. Robert Underwood <underwoo@u.washington.edu> 13 Oct 2005

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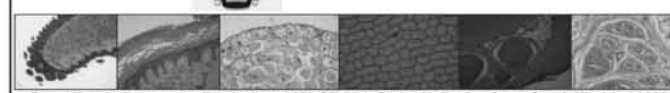

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We have very consistent results with a different protocol. Check it out on our website (protocol 7): http://cellserv.med.yale.edu/imaging/ccmi/elect_protocols.html. The main differences are: - We use 0.1 M NH4Cl instead of glycine, but that should not matter much. - We use 1% of fish skin gelatin instead of BSA. - We use PBS instead of TBS, but again that should not really matter - Incubations with antibodies and protein A-gold are for 30 min. This could be an important factor. Why do you incubate so long with antibodies? Maybe when antibodies don't work too well it would make sense, but I wouldn't do this systematically. This is bound to create problems with aggregation of antibodies, increased background, etc. Also, we only block with NH4Cl and fish skin gelatin for a maximum of 10 and 20 minutes respectively. This means most of our immunolabelings are completed within 2 hours! Much more cost-efficient I would say. Marc Pypaert <marc.pypaert@yale.edu> 13 Oct 2005

COLLOIDAL GOLD - conjugation with fluorescent BSA

I am trying to conjugate my colloidal gold particles (0.8 nm and 6 nm particle sizes) with fluoresceinated BSA. I have tried to perform extensive literature search for detailed protocols but most of what I found were either for specific proteins (like IgG or Protein A) or general recipes that don't really say much about what I should do. I know that we have experts out there who have done such procedures (or others that may be very similar) in the past; any advice/information would be greatly appreciated. Carlo Franco Bolivar Balane <cbalane@wesleyan.edu> 19 Nov 2005

Most colloidal gold conjugation protocols are for antibodies or other targeting agents. BSA is used as a stabilizer in these reactions, but because conjugation of the targeting agent takes priority, the reaction conditions are usually optimized for conjugating to this rather than the BSA. However, you don't need to make many changes - the procedure is similar for most colloidal gold conjugations. Optimum colloidal gold conjugation is usually done at a pH at or just above the pI of the protein you are conjugating. From a quick search, the pI of BSA is about 4.7, so a pH of about 5 to 5.2 would be good for conjugation. Usually you would dialyze the protein into a dilute buffer at this pH or deionized water; use a buffer that does not flocculate colloidal gold (for example 0.002 M citrate, pH 5). Adjust the pH of the colloidal gold sol to the same pH using dilute H3PO4; then add the minimum stabilizing amount of fluorescein-BSA to the gold (you can determine this from titration; a procedure is given in the link below), stir for 2 minutes, then add a further 10% of BSA and 0.1% Carbowax (a special form of 20,000 MW polyethylene glycol), stir 10 - 15 minutes more. A good procedure for antibodies is given in this link. To adapt for albumin, all you need to do is to change the pH for the titration and conjugation to one more suited to BSA (adjust pH with H3PO4 if you need to lower rather than raise it): http://www.researchd.com/gold/gold8.htm. All glassware must be scrupulously clean. Glass and plastic containers and stirrers should be cleaned in aqua regia, thoroughly washed in deionized water, and siliconized. Once you have prepared the conjugate, you should store at a pH several units away from the PI, as this helps stabilize the conjugate. Spin down and resuspend in 0.02 M Tris-HCl, pH 8.2 (the usual buffer for storing colloidal gold conjugates). Before doing this, though, you should be aware that fluorescence will likely be completely quenched if you conjugate fluorescein-streptavidin to 6 nm gold - gold is a very efficient absorber for resonance energy transfer, and other mechanisms may also be present that will quench the fluorescence even more than energy transfer alone. We have explained this, and Albrecht and co-workers have observed it in practice when making combined fluorescent and 6 nm gold-labeled antibodies: (1) Powell, R. D.; Halsey, C. M. R., and Hainfeld, J. F.: Combined fluorescent and gold immunoprobes: Reagents and methods for correlative light and electron microscopy. Microsc. Res. Tech., 1998, 42, 2. (2) Kandela, I. K.; Meyer, D. A.; Oshel, P. E.; Rosa-Molinar, E., and Albrecht, R. M.: Fluorescence Quenching by Colloidal Heavy Metals: Implications for Correlative Fluorescence and Electron Microscopy Studies. Microsc. Microanal., 9, (Suppl. 2: Proceedings); Piston, D.; Bruley, J.; Anderson, I. M.; Kotula, P.; Solorzano, G.; Lockley, A., and McKernan, S. (Eds.); Cambridge University Press, New York, NY, 2003. Rick Powell <rpowell@nanoprobes.com> 20 Nov 2005

IMMUNOCYTOCHEMISTRY – GFP and Triton X-100

Does anyone know if GFP fluorescence survives when formaldehyde-fixed tissues are permeabilized with Triton X-100? I need to double label a GFP expressing tissue. Tom Phillips <phillipst@missouri.edu> 01 Dec 2005

I would use an anti-GFP and do the labeling that way. There may be some GFP left after that treatment, but I would not count on it. GFP antibodies will work well. David Elliot <elliott@arizona.edu> 01 Dec 2005

I'd say it depends on your construct; I've done Triton X-100 permeabilizations on cultured cells that were expressing GFP fused to non-extractable proteins and had no (detectable) loss of fluorescence. Tamara Howard <thoward@unm.edu> 01 Dec 2005

In my experience, GFP is a robust signal, and survives most insults including permeabilization; the exception being hypoxia for an extended period. The main concern I would urge you to consider is signal overlap. If your signal from your GFP is robust as I would expect it, you will have bleed-through into the red channel. I have gotten around the issue by using far-red fluorochromes. Elizabeth Henson <alpha79@mts.net> 01 Dec 2005

IMAGE ANALYSIS – quantitative comparisons

A researcher has asked me to find out if it is reasonable to do anything like a quantitative comparison between fluorescence images of different samples. She has thick sections, 20 µm, on a confocal scope. She sees differences by eye between control and treatments, and she would like to quantify these differences in some way. We have thought about setting up the confocal to record the brightest image, then without changing the settings, record an image of the other slides. The idea would be that she could do something like compare the brightness levels between them. I don't know enough about other options or if this will even work to help her. Does anyone do anything like this or is this not realistic? Jonathan Krupp <jmkrupp@cats.ucsc.edu> 01 Dec 2005

The approach you describe to this problem would be sound if all other factors were controlled and, in fact, we use it sometimes. The problem with quantification of fluorescence is that there are so many parameters to control and there is the non-linearity in fluorescence emission to consider, i.e. is emission intensity linearly related to the amount of fluorochrome present? Assuming that physical parameters (preparation technique, section thickness,

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stain concentration, fluorescent protein expression levels, etc.) are controlled, you can fairly easily say that one specimen is dimmer than another. One of the most important caveats is to ensure that the emission levels in the brighter image do not exceed 255. If they do, then you can not reasonably say how bright the brightest point really was. Use a 'glow' palette in the confocal software when setting gain levels to ensure that the brightest specimen levels remain just below saturation. What do you do next? The real trick is to describe why one specimen is brighter than another. Either it will have to do with the relative amounts of the stained tissue or the relative levels of gene expression. Both are developmental problems that can be solved genetically or biochemically. John Runions <jrunions@brookes.ac.uk> 01 Dec 2005

Any comparison between images is complicated, as in most cases the acquisition conditions are not or can not be controlled so as to give you identical conditions. This is even more difficult in fluorescence images, as the fluorescence can bleach over time, so not only the conditions, but also the timing must be right. The first problem you can try to tackle by embedding some standards into the preparation. Perhaps some small fluorescent beads that you can then use to normalize the image. Instead of using the intensity itself, you would use the intensity ratio of sample/bead as a measure. If your samples bleach, you can try to do several things: 1) acquire a time series of each sample and measure the decay of the signal and extrapolate to time zero, or 2) acquire the image at precise times, for example 1 minute after turning on the illumination. Mike Bode <mike.bode@soft-imaging.net> 01 Dec 2005

TEM – hepatitis C infected tissue fixation

A graduate student is planning to fix hepatitis C infected human liver tissue for TEM (fixation and embedding) work. I would like to know what would be the best way to fix the fresh tissue and transport it from a hospital. I am also interested to find out what precautionary measure we should take since the student will be handling infected liver tissue. We are planning to fix in glutaraldehyde in cacodylate/phosphate buffer. Is the virus considered infectious after fixation? Any suggestion will be greatly appreciated. Soumitra Ghoshroy <sghoshro@nmsu.edu> 07 Nov 2005

Your question was outside my experience, so I contacted a virologist/microscopist I know, and she kindly sent me the following. The required precautions sound quite serious and she suggested you contact her with any questions. Response: Any clinical specimen should be handled with "Universal Precautions". There is a set of guidelines known to anyone who routinely collects potentially infectious material. I would let this person place the tissue into fixative. These procedures entail the use of gloves, lab coat, face protection, special procedures for not cutting or sticking oneself with sharp objects, and use of a biosafety level 2 cabinet. Whoever obtains the specimen from the patient can place it directly into glutaraldehyde for transport. This will render the specimen non-infectious since the agent is an enveloped virus (flavivirus). Prion material is not killed by aldehyde fixation, but all human viruses are. Ask the person obtaining the sample to cut it into small pieces or slivers if possible (a few mm). If you get a large chunk (e.g., a cubic cm), let it sit in glutaraldehyde overnight to ensure that the fixative gets inside. Then take your 1 cubic mm slices for EM from the surface that is well fixed. The center may or may not be well

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fixed, depending on how long the tissue sat before being placed into the glut. At this stage, you can handle the tissue just like any other non-infectious specimen. Note that few good micrographs of HCV have been published, particularly from infected human liver. Also, note that flaviviruses are small (40-60 nm) enveloped RNA viruses, and that other things in the cell can resemble these particles. Most diagnoses of HCV are made histologically on the appearance of the liver, by immunostaining, and/or serologically, but not by EM. Sara E. Miller via a communication by Mike O'Keefe <maokeefe@lbl.gov> 08 Nov 2005

TEM - embedding problem

Lately when I embed clinical tissue in blocks I get tiny little holes all over the tissue. I changed the dehydration ethanol and propylene oxide. Do you have any suggestions? Kathryn Privett <morgansbear-hunter@yahoo.com> 28 Nov 2005

My guess is, the holes will go away if you use a dehumidifier to bring the relative humidity in your work-room (or work-chamber) below 60-70% before, during and after the last change of 100% accelerated resin. We used to get episodes of holes in thin sections of our Araldite 506-DDSA-DER 736 resin mixture until we followed the advice we found in this paper: (1977) H. D. Dellman and C. Pearson Better epoxy resin embedding for electron microscopy at low relative humidity. Stain Technol. 52:5-8. This is essential for us, perhaps more so than for most labs, because we need a dehumidified environment during the 15-90 minutes it may take us to lovingly manipulate fully infiltrated single muscle fibers and rafts of 3-5 single fibers into position on regions of dry substrate with only a minimal micro-meniscus of external resin; once thus positioned, they stick themselves nicely to each other and to the smooth substrate of polypropylene sheet (by surface tension) during the first 2-4 hours of cure at 60° C. We then invert a full BEEM capsule of liquid resin over the fiber or raft and finish the cure overnight at 80°C. This gives us superbly oriented single fibers exactly parallel to the flat surface of the cured block and lying within 3-5 microns of the resin surface; we like to use it to co-embed fibers from different experiments in a single raft, so we can make one longitudinal (or cross-) section of a such a combi-block do the work of 3-5 sections of separate blocks. The very high surface-to-volume ratio of this "dry, flat" embedding procedure makes the resin very susceptible to humidity during the pre-cure manipulations. So we go for as low a humidity as our commercial dehumidifier can achieve in our small workroom, often below 50% if we run the thing all night in a closed room. Obviously even lower levels could be rapidly attained if the dehumidified outflow from the appliance was delivered into a small desktop working chamber, but we've not found that to be necessary for our work. The curing oven itself is not a worry-- its internal RH is below 10%, if I recall our few measurements some years ago. So we typically accelerate infiltration itself in 100% accelerated resin mixture by putting the vials on a sloped rotator in the 60°C oven, and remembering to change the resin for fresh every 30 minutes, x2 or x3. In that heat, the resin mixture becomes so water-thin, and stays that way for at least 40 minutes, that I've long supposed even Spurr's or similar could not become significantly lower in viscosity or penetrate the tissue any more completely. Mike Reedy <mike.reedy@cellbio.duke.edu> 28 Nov 2005

TEM - Plan view sample prep contamination

We met some contamination problem when preparing plan-view TEM sample where the sample can only be milled from one side. We found that the contamination at the unmilled side is quite significant (re-deposition). Will the graphite post help? Is there any other procedure to minimize the contamination? Simon Lee <kunli218@yahoo.com> 15 Nov 2005

I have a PIPS which I use for cross sections and the occasional plan view. I find it is not too bad, but I usually have to give the original surface a tickle with the ion beam to clean off contamination. I generally use the standard double-sided holder, not the graphite post (since I have never been very confident about getting the sample off the post again without breaking it!) Milling conditions are double modulation, 3 degrees incidence from below with the top surface up (i.e. facing the viewing port), slowest possible rotation, 6kV. When the sample is thin enough I turn the voltage down to 2.5 kV (the gas flow has to be adjusted to get maximum current) to give a final clean of the milled surface for a minute or two. The top surface clean is also at 2.5 kV but only one burst of the gun, probably about 12 seconds. This has worked quite well; I have identified nm scale contamination on SiO_2 films without any real problems with artifacts. It probably helps to stop milling as soon as you get the smallest hole. If I really have to keep the top surface intact I prefer using chemical methods, jet etching with Cl in methanol for III-Vs or HF:HNO3 for Si. <richard.beanland@bookham.com> 15 Nov 2005

For plan view samples I prefer to use the regular post sample holder. I attach the sample with a tiny drop of crystal bond (I mean tiny!), which then gets cleaned off manually with a pointed q-tip and acetone. I do not like to soak off the samples because I feel it contaminates the area you just milled. Also, stop milling when you get the smallest possible hole or it will redeposit through the hole, and reduce the kV as you get closer to finishing. If crystal bond residue cannot be tolerated (of course you can't clean it all off), then I use the graphite holder, with a 3mm disc cut from a glass coverslip underneath the sample to protect the surface. The only problem with the graphite holder is the slider bars on either side will block the beam when using rotation only, and the hole will end up somewhat oblong. If none of that works, you can mill the sample for a few seconds on the 'good' side, but this is hit or miss. Leslie Krupp (Thompson) <lkrupp@us.ibm.com> 15 Nov 2005

There are a couple of ways to prevent contamination on the back side during ion milling of a plan view sample. The most common way is to use a lacquer that you remove afterwards. I can't find the reference for the neatest way that I think is the way to do this. I thought that it was in the first MRS TEM sample Prep book (Vol 115), but I couldn't find it there. What you do is evaporate NaCl in a vacuum evaporator onto the side that you want to protect. Ion mill on the other side. The NaCl layer is the one that is contaminated and after perforation in the ion mill, you simply dip the sample in distilled water to dissolve the NaCl and float off the contamination layer. I wish that I could remember who did that, but I thought that it was a pretty slick idea. If anyone knows the reference or who did it, please let me know because I would like to put that in our application notes section of our web site. Scott D. Walck <walck@southbaytech.com> 15 Nov 2005

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TEM - contrast and focus

I've just been looking at some thin crystals of an organic compound, sitting on a carbon film, under bright-field TEM. When in exact focus, they appear much less contrasty than when the focus knob is turned to the left of the right. When turned to the right, dark fringes appear around the crystals. I would like to ask: (1) Which of the two, left or right, is over/under focus? (2) Why are they more contrasty overall when over or under-focused? (3) Why are they more contrasty at lower magnification? Robert H. Olley <hinmeigeng@hotmail.com> 24 Nov 2005

When you turn to the right and see a dark fringe at the edge of objects then it is overfocused and a light fringe is underfocused when you turn to the left. These are Fresnel fringes and are produced because of diffraction contrast, which is a result of scattering of light/electrons at edges. It can and is quite often acceptable to defocus slightly to increase contrast but this should normally only be slightly underfocused. The logic being that bright fringes will enhance the dark edges beside them, but dark fringes will introduce more apparent dark structures. When you examine specimens at low magnification amplitude contrast makes a greater contribution to the image than phase contrast. Amplitude contrast is generated by the influence of the specimen's atomic nuclei on electrons in the beam. The higher the mass of the nucleus the greater the 'scattering power' of that area of the specimen and the more electrons scattered out of the main beam path the darker that area. Amorphous samples simply produce more or less contrast by mass alone although crystals scatter electrons in much more defined ways due to the interaction of the 'wavelength' of the electrons, the lattice spacing in the crystal and its angle of tilt. There are many good books on this subject, but here's the first one that came to hand: Principles and Practice of Electron Microscope Operation; A.W. Agar, R.H. Alderson and D. Chescoe; Pub North Holland (3rd print 1980) ISBN 0 72044255 9 where chapter 3 on image formation is particularly appropriate. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 24 Nov 2005

Your description is right on target. When I was learning to use the EM, I was taught to obtain best focus by experimenting first with a holey sample so that I could adjust what my eyes thought was best focus (because we are uniquely sensitive to contrast) to the true focus. I then would focus on a sample to what I thought was best, and then adjust the focus setting by a few notches. The issue of whether you want to include the underfocus fringes in your image becomes, then, an issue of whether you want to include the optical artifact for contrast, or you are interested in the "true" edge of your objects. Joel Sheffield <jbs@temple.edu> 24 Nov 2005

I might be wrong, but seem to have a little different understanding from the posts so far to these concepts of very importance in both theory and practice so that I reply, hoping to solicit more insights. In short, 1) Slight under-focus (OL knob counterclockwise adjustment from the least contrast or true focus point) not only makes the image look good, but provides better resolution. 2) There are only two types of contrast mechanisms in my opinion: amplitude contrast and phase contrast. Amplitude contrast includes a) diffraction contrast, b) mass-thickness contrast, and c) Z-contrast (a special case of mass-thickness contrast). Also due to the locality nature, Z-contrast and phase contrast can normally be thought responsible for or capable of providing lattice resolution (you may argue with in the cases of imaging with several diffraction spots) or atomic resolution. Chaoying Ni <cni@udel.edu> 24 Nov 2005

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As far as I can see, all of the contributed points of view are correct. Underfocus by a few hundred Angstroms will indeed enhance contrast and improve resolution. The explanation was first given by Otto Scherzer in his famous "20/20" paper on page 20 of volume 20 of J. Appl. Phys. [Scherzer, O. (1949). "The theoretical resolution limit of the electron microscope" J. Appl. Phys. 20, 20-29]. Scherzer describes how an underfocus of minus sqrt (*Cs* * wavelength) optimizes transfer of spatial frequencies into the image from the electron wave leaving the specimen -- by balancing (positive) phase shifts due to spherical aberration with (negative) shifts from underfocus to form a kind of quarter-wave plate. Optimum underfocus ranges from -750 Angstrom for a spherical aberration of 1 mm at 100 kV to half that for a *Cs* of 0.5 mm at 300 kV. Be aware that too much underfocus (or overfocus) will add too much negative (or positive) phase shift and "scramble" the image so it no longer shows a simple projection of the specimen. There is more on high resolution and focus in many publications -- the one I like is the paper "Resolution in high-resolution electron microscopy", M.A. O'Keefe, Ultramicroscopy 47 (1992) 282-297. For TEM of organic molecules see papers by John Fryer -- especially J R Fryer 1993 J. Phys. D: Appl. Phys. 26 B137-B144. There's also M.A. O'Keefe, J.R. Fryer and D.J. Smith, Acta Cryst. A39 (1983) 838-847. Michael A. O'Keefe <maokeefe@lbl.gov> 24 Nov 2005.

You might want to have a look at a few useful webpages: a focusing simulator: http://www.umsl.edu/~fraundor/epc/index.html and contrast transfer function: http://clik.to/ctfexplorer http://ncmi.

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bcm.tmc.edu/homs/wen/ctf/ctfapplet.html. Philip Koeck <philip.koeck@biosci.ki.se> 25 Nov 2005

SEM - Vibrio parahaemolyticus samples

I'm trying to prepare Vibrio parahaemolyticus samples for SEM on 2216-marine agar plates and am having difficulties visualizing the bacterial cells on the agar surface. I have used a 1% glutaraldehyde/ 0.1M cacodylic acid plus 3.5% salt fixative and fixed cells by gently pouring over the agar plate. It appears as though cells are somehow diffusing or migrating into the agar and avoiding being fixed directly on the surface. Any help would be appreciated. Emiley Eloe <eeloe@ucsd.edu> 24 Oct 2005

Carefully cut out 1 millimeter x ~10 mm x ~1 mm pieces of the agar where the bacteria are. Fix for a couple of hours at room temperature (or overnight in the refrigerator) and dehydrate as usual. After the final 100% ethanol step, drop the pieces into liquid nitrogen. The ethanol freezes vitreously, and so won't cause any freezing artifacts. If the pieces don't spontaneously fall apart, break them with a razor blade. This will expose the bacteria in the agar. This method also works for all sorts of things. Return to 100% ethanol, then critical-point dry. Phil Oshel <oshel1pe@cmich.edu> 25 Oct 2005

The advice from Dr. Oshel does not solve the problem that the bacteria would not stay together during fixation in aqueous glutaraldehyde. When I wanted to see the surface of a colony, I fixed the small pieces of the agar gel with the colonies on them in osmium tetroxide vapor in a humid chamber and then carefully dehydrated them in a graded ethanol series. If the original writer only wants to get images of the bacteria irrespective whether they are from the top or the bottom of the colony, it would be advisable to gently release (from a Pasteur pipette) a thin layer of >40°C 3-4% agar sol. I used to incorporate glutaraldehyde into the agar sol before use. This coating would immobilize the bacteria in the colony and the sample would be dehydrated and critical-point dried as usual. The dried agar coating would be opened easily before mounting the bacteria on a stub. (Response from a retired scientist communicated by Ann Fook Yang). Ann Fook Yang <yanga@agr.gc.ca> 26 Oct 2005

It won't? Odd. I've used the fix-ethanol cryofracture methods many times to study bacteria and yeast within (and on) agar without the critters "not stay[ing] together". The glutaraldehyde fixation step fixes the bacteria in place quite nicely. Phil Oshel <oshel1pe@cmich.edu> 26 Oct 2005

SEM - imaging of bacteria encapsulation

We have a student working on a project in which they are growing bacterial cultures (Pseudomonas and other species) in a hostile environment. The bacteria form capsules that we are interested in seeing. The capsules are most likely composed of sugars (polysaccharides), and we are hoping to find a fixation or other preparation technique that would help us with imaging these. We have so far prepared cultures with standard fixation, dehydration, and critical point drying techniques, but are not sure that we are preserving the capsules forming around the bacteria. We do not have a cryo stage available. Any ideas? Karl Hagglund <hagglundk1@nku.edu> 14 Nov 2005

You might try using some Alcian blue in the primary fix to stabilize the capsules. It works for us for TEM with Campylobacter. Chris Jeffree <c.jeffree@ed.ac.uk> 14 Nov 2005

There are "non-aqueous" fixation techniques and methods involving ruthenium red and Alcian blue that are useful in preserving these capsules and other biofilms. The first method involves dissolving osmium tetroxide in a solvent, such as FC-72 from 3M Company, rather than in an aqueous buffer. For details, see Microscopy Research and Technique 36:390-399 (1997) and 36:422-427 (1997), Biotech Histochem 66(4):173-80 (1991), and J Comp Pathology 117(2):165-70 (1997). Randy Tindall <tindallr@missouri.edu> 14 Nov 2005

Another proposal: tannic acid, low molecular weight (approximately MW 1600), applied either in the fixative (0.05 - 0.1 -1.0 %), be sure your glutaraldehyde is of EM-grade, or fix as you like (buffered as usual, including glutaraldehyde, perhaps higher concentration = 3% to 6%), wash as usual, postfix with OsO_4 (buffered, 1-2% as usual), start dehydration with 50% ethanol, and immerse then with 1% para-phenylenediamine (PPD, use with care according to MSDS) in 70% ethanol for at least 30 - 60 min, wash the specimens several times in pure 70% ethanol (to get rid of unbound PPD) and go on with dehydration/embedding as usual. I have not tried acetone as a dehydrating agent, but I don't see a reason why acetone as a dehydrating agent should fail. There will be an interesting difference in morphology, as compared to "normal" fixation and dehydration. You could also add a tannic acid (0.05%-0.1 % hydrous solution, filtered) pre-incubation staining of the ultrathin sections grids before the classical two-step staining procedure with uranyl acetate and lead citrate. Wolfgang Muss <w.muss@salk.at> 14 Nov 2005

Saccharides are seen in very high contrast with the use of ruthenium red. It is described in some of the older literature by Luft, I think. Carol Heckman <heckman@bgnet.bgsu.edu> 14 Nov 2005

Freeze drying might be the best bet for holding on to everything without adding artifactual material such as ruthenium. Gregory Erdos<gwe@ufl.edu> 14 Nov 2005

Two possibilities are negative stain and cryo-fixation. Even though you do not have a cryostage, high-pressure freezing followed by freeze-substitution and embedding will give you the specimen in a block of resin that can be stained and sectioned in the conventional manner. Of course, you may not have access to a high-pressure freezer. Bill Tivol <tivol@caltech.edu> 14 Nov 2005

You might like to try a simplified cryo-fixation method. It is not the best method for many reasons and may not give you an impressive result as the high-pressure freezer that Bill Tivol suggests, but it will give you an easy way into these methods. Hopefully the results will give you enough data for a grant! First freeze your bacteria by immersion freezing in as close to the growing state as possible. If they are growing in suspension, spin them down very gently and scoop out the pellet to place onto a metal holder (any small piece of wire or metal that can hold a drop of the suspension will work). If they are growing on a substrate, leave them there. Take the bacteria and freeze them by immersion in an efficient cryogen. We use liquid propane from a bottle we get from a camping store (for cooking food outdoors) - disclaimer here: propane is inflammable so take suitable precautions not to blow up the lab. We collect the propane by piping it into a small plastic cup that is surrounded by liquid nitrogen. Once the bacteria have been frozen, which is almost immediately after they have been immersed in the propane, transfer them to vials on dry ice, containing dry ethanol or acetone. The dry ice is held in a Styrofoam box. Leave them on the dry ice for a few days (depending on the size of the specimen), changing the cold solvent with fresh cold solvent every day. Transfer the specimens to

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the refrigerator (4°C) in a small Styrofoam box containing a small amount of dry ice and leave the specimens to warm to 4 degrees. The trick is to let the vials warm up slowly enough to not have the solvent boil. Once you have the solvent at 4°C, the specimens can be transferred to a critical point drier and processed for SEM examination. As I said, this is not the best way to prepare specimens using cryomethods but you may be surprised by the results you get. Paul Webster <pwebster@hei.org> 14 Nov 2005

Just to offer a heretically simplified option for quick-and-dirty (but often unexpectedly clean) speedy results: I agree with Muss that tannic acid is great, the surest simple fixative, and with Webster that freezing is a promising approach. But I suggest that for fixing extracellular matrix, and intracellular structures of detergent (Triton X-100) pre-permeabilized cells, you can do quite well without OsO_4; just use freshly dissolved 0.2% tannic acid in your physiological extracellular or intracellular buffer at pH 6.8-7.0 (absent any components that precipitate in tannic acid) for 30 min, then wash out all unbound tannic acid in 5 buffer changes, and follow with 1% uranyl acetate in deionized water, no pH adjustment needed, then do acetone or ethanol dehydration as fast as you like. If you wish, you can interpose 0.5-2.0 hr in 1% glutaraldehyde after the tannic acid and before the uranyl acetate, but I usually use straight tannic acid – uranyl acetate as a preferred binary fix; it is certainly less toxic and just as fixing as a similar tannic acid-osmium procedure. (M. K. Reedy, C. Lucaveche, D. Popp (1991) Biophysical Journal 59, 579a). Worth one shot for comparison is using a tannic acid-glutaraldehyde mixture as a trial primary fix before uranyl acetate, but I find glutaraldehyde sometimes tends to rush in and perturb orderly lattice arrays that tannic acid alone fixes more slowly and beautifully. Tannic acid primary fix must be stabilized by uranyl acetate or OsO_4 secondary fix before dehydration. I've used the same tannic acid – uranyl acetate sequence in cryo-acetone for freeze-substitution almost exclusively for 15 years. That's what suggests to me that it is possible value for polysaccharides. It is able to fix and stabilize (or perhaps it just permits physical in-situ stabilizing of) the threadlike molecules of 500,000 MW dextran we sometimes use at 3-5% as an osmotic squeezer for the myofilament lattice of glycerinated muscle fibers. We can see them in thin sections, excluded to the space between myofibrils. However, the aqueous tannic acid – uranyl acetate fix does not preserve /retain the free dextran molecules, so I guess the quick-freezing and/or acetone is needed for that. So I guess maybe using an initial non-cryo "fix" of acetone alone, followed by tannic acid – uranyl acetate in acetone and comparing with acetone- tannic acid – uranyl acetate sequence and with an acetone-uranyl acetate-tannic acid sequence might be worth trying. Note Craig lab's findings that uranyl acetate alone can be a fabulous fixative. (F. Q. Zhao, R. Craig, Journal of Structural Biology 141, 43 (Jan, 2003). And if you want to try a totally safer alternative than propane for the plunge-freezing that Paul Webster suggests, LN_2 plunging has a bad rap that is undeserved. If you plunge rapidly through 10-12 inches of LN_2, you leave behind all the insulating bubbles responsible for the Leidenfrost effect and get convective cooling at least as rapid or more-so than propane provides. See what protein crystallographers from Hope's lab have

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to say about how reliable this can be! (L. J. Walker, P. O. Moreno, H. Hope, Journal of Applied Crystallography 31, 954 (1998); S. Parkin, H. Hope, Journal of Applied Crystallography 31, 945 (1998)).Mike Reedy <mike.reedy@cellbio.duke.edu> 17 Nov 2005

SEM – gold films

I've seen many questions about grain size of sputtered gold on this list, but what about the tendency of gold films to break up into a sub-micron "crazy-paving" structure? Does anyone know what causes this, and how to cure it? Often the crazy paving totally dominates the picture, and one has to mentally filter it out to see the underlying structure. Robert H. Olley <hinmeigeng@hotmail.com> 28 Nov 2005

One cause of the "crazy-paving" or "dried river bed" appearance of specimens that were coated with heavy metals for SEM viewing is the expansion and contraction of the underlying specimen. Since the metal coating has little flex/stretch capabilities, it will break or shatter if the underlying specimen flexes or expands. The causes of the expansion of the specimen may be: absorption/loss of moisture as one goes in and out of the vacuum, heating/cooling, or mechanical flexing. This can be minimized by keeping the specimens always in a dry environment, at a standard temperature and not bending/flexing them. John J. Bozzola <bozzola@siu.edu> 28 Nov 2005

The quick answer to your question concerning the structure of gold coatings is to not use gold coatings. Gold is notorious for nucleating islands on the surface that grow and eventually coalesce to form the coating. This growth pattern results in your "crazy-paving" structure. In fact, the resolution test sample for SEM has traditionally been a gold coating evaporated onto a smooth carbon substrate and the minimum distance between adjacent gold islands is used as the acceptance measurement for the microscope. Au-Pd will offer a better coating on inexpensive desktop coaters than plain gold, but you will still see the grain structure at higher magnifications. Ion Sputter coating systems can sputter numerous different materials. The materials that offer extremely good, uniform, and small structure suitable for high resolution SEM work are Pt, Pd, Cr, Ir, and W. Scott D. Walck <walck@southbaytech.com> 28 Nov 2005

Our philosophy has always been that if this is happening, then you are probably putting on too much gold and/or exposing the sample to too much heat. Other factors can also increase the chances of seeing this kind of effect, for example, the presence of a thin lubricant coating such as on a storage media surface, catheter tubing or syringe needles. Additionally, certain materials like PTFE are especially prone to this kind of cracking. I have always assumed that this was at least in part due to the inherent "grain" structure of any sputtered coating. A layer of osmium metal deposited in an OPC osmium plasma coater, which is not a sputtered coating, has no grain size (at least no one has detected a grain size to our knowledge) and seems to be much more resistant to the "crazy-paving" structure pattern effect on the above mentioned samples. I propose that the osmium coating approach would qualify for the requested "cure". Charles A. Garber <cgarber@2spi.com> 28 Nov 2005

I've seen this "spider man" appearance before and attribute it to sputtering at too high of pressure and too high of current. Gold is not a great metal for high resolution SEM anyway. Au/Pd is better, and then consider Pt or Ir. But it seems to me that low vacuum (15mT) and low current (9mA) makes a big difference. But you will not likely get 15mT or lower without a turbo pumped system. Try your system at the lowest vacuum you can get and see what happens. Gary Gaugler <gary@gaugler.com> 28 Nov 2005

EDX - Aperture vs Current

What would be the benefit of using a larger objective aperture instead of increasing the probe current in order to increase the beam current for EDX analysis and X-ray mapping? Willem Wennekes <willem.wennekes@comcast.net> 02 Nov 2005

The benefit is that you keep your small aperture clean for imaging. David Hull <david.r.hull@nasa.gov> 02 Nov 2005

When you increase your probe size (demagnify the source less), the spray electrons from each crossover tend to be captured closer to the beam path thereby creating heavier contamination buildup nearer to the beam. This can result in faster degradation of your imaging capabilities and the need for more frequent cleaning of the column. Ken Converse <kenconverse@qualityimages.biz> 02 Nov 2005

What are the options for increasing probe current? i.e., What things can you do to increase it? Yes, increasing the aperture size will increase probe current. However, it increases probe diameter. Another option is to increase kV. This will greatly increase volumetric interaction such that more information comes from deeper in the specimen. It is a constant trade-off problem. Before changing anything above, be sure your specimen is at the optimal analytical working distance (WD) for the EDS. If you don't have that value, you can find it by experimentation. Just start at a relatively long WD, check the counts per second and then start reducing WD. Keep checking the cps at each successive reduction in WD. The cps will start to increase and then decrease. The WD at max cps is your analytical WD. Work at this WD. Now, back to aperture size and kV. I'd start with kV first. Based on the highest Z you are looking at, you will need about 2X the eV of the line value you are collecting. Look at the M, K and L series values and determine the necessary kV. e.g., Aluminum. Z=13. No L-series lines. $K\alpha$=1.486KeV. $K\beta$=1.553KeV. No N-series. So, K-series are the only ones ($K\alpha$ is the one used for calibration along with Cu $K\alpha$ at 8.040keV). So for Al, 5kV would probably be a good value. If the specimen is bulk, then higher kV is OK and would increase probe current and cps. Take an extreme such as W (Z=74). Here, we have lines at all shell series. However, not all of them are practical for normal SEM. $K\alpha$=59.305KeV (can't use this since kV would need to be about 120KV). Same problem with $K\beta$. $M\alpha$=1.774 keV. Sorting through the other lines, $L\alpha$ pops up at 8.394 keV. So 2X this would be 16kV. So, 18-20kV would be appropriate. But at this kV, volumetric interaction would be very high. High Z and high kV will result in skyrocketing cps. So, a smaller aperture would be necessary to keep dead time down to <30% or so. If you want to quant, this is probably the appropriate condition. However, for mapping, if you do not have a volumetric interaction situation, and you do not need high resolution, you can increase kV and aperture size to increase cps. Keep in mind that most EDS systems need cps to be such that dead time is ≤35% or thereabouts. Increasing cps with resulting higher DT simply throws away data since the pulse processor cannot handle the high number of counts. Each system has their own limit on this. Just look at DT to be sure the processor is not overloaded. Again, if the specimen is bulk versus very small, then increasing aperture size will increase probe current and cps at the expense of resolution. But for low magnification, bulk specimens, not much of an issue. Hope this

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helps. Gary Gaugler <gary@gaugler.com> 03 Nov 2005

I think there are two issues here. One is the "physics" question (what does theory say?) and the other a practical question (what happens in an actual instrument operating in a normal way?). First, the physics. Here the ultimate limit is diffraction. Regardless of the source type, a too-small aperture will cause a diffraction spreading of the beam. The simplest way to understand this is as a manifestation of the Heisenberg uncertainty principle -- when the lateral location of the electron is too tightly constrained by the aperture, then the lateral uncertainty in the momentum increases and there is broadening. So if one were operating a SEM in a diffraction-limited mode, increasing the aperture size would be the most sensible way to increase the beam current. Most thermionic (not FE) SEMs are not operated anywhere near the diffraction limit. Usually the dominant limit to resolution is spherical aberration and that term varies as aperture size to the third power. The simple quadrature approximation shown as equation 2.14 in Goldstein (2nd Ed) is generally regarded as valid for thermionic SEMs, but is not valid for any instrument operating near the diffraction limit. Here one must use much more complex wave-function calculations -- it's been a long time since I did this, but the role of the aperture is the same. There is an optimum aperture size for any given beam current and either a smaller or larger aperture than that optimum will increase the beam diameter. Now for the practical. What actually happens when you turn a knob or change an aperture on a particular instrument depends on the details of how that instrument was designed and is being operated. When a SEM employs a "virtual" aperture (the physical aperture doesn't reside in the principal plane of the probe-forming lens) then it is possible to change the *effective* aperture size by manipulating the condenser lens(es) and thus the effective aperture size can be optimized by the control software to be optimal for that probe current without changing the physical aperture. Even if that is not the case, it's usually impractical or inconvenient to optimally match the physical aperture to its ideal size (apertures tend to be available in rather coarse diameter steps). Consequently, for modest changes in beam current, just turning the "Spot" control (or whatever you call the control that regulates the condenser current) is the simple practical expedient. But in more extreme cases, where one wants to drastically increase the beam current but doesn't want to unduly enlarge the spot, one is well advised to pay attention to the physics -- the principle works. Now, back to the original question that was asked. "What would be the benefit of using a larger objective aperture instead of increasing the probe current in order to increase the beam current for EDX analysis and X-ray mapping?" There are certainly many situations where choosing a larger aperture size will permit the higher probe current required for x-ray analysis to be achieved with minimal degradation of beam diameter. That is a practical reality in many situations. There may be other good reasons, and this answer won't apply to all situations. But it is a mechanism that needs to be considered given the limited information provided. So far from being a "nuance," depending on the instrument being referenced, this may in fact be the most pertinent answer to the question posed. Fred Schamber <schamber@aspexllc.com> 03 Nov 2005

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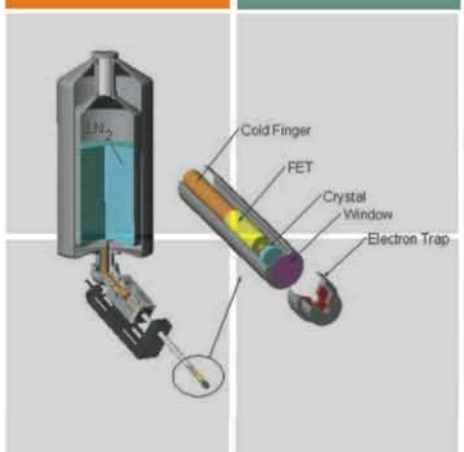

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