NETNOTES

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Selected postings from the MSA Microscopy Listserver (listserver@msa.microscopy.com) from 02/15/06 to 04/15/07. Postings may have been edited to conserve space or for clarity.

SPECIMEN PREPARATION - LR White polymerization

I am new to LR White. The instructions for "Electron Microscopy" strongly recommend curing 20-24 hrs at 60°C ± 2°C and warn of over brittle blocks if this is not followed. But, the instructions for "Electron Microscopic Immunocytochemistry" recommend 50°C for 24 hours. My clients want LR White embedding for immunogold staining. I would appreciate feedback from regular users as to what temperature and curing time they use. Ralph Common <rcommon@msu.edu> 12 Mar 2007

We got it to polymerize at 52-53°C for 24 hrs. Worth trying 50°C with a blank block. We did not notice any sectioning problems. Dave Patton <david.patton@uwe.ac.uk> 12 Mar 2007

Worthwhile bearing in mind the possibility of protein/antigen extraction during long infiltration periods in LR White and during slow polymerization at 50°C. I and others have found that using LR White accelerator (1.5 µl per ml) and immersing the molds in a crushed ice slush to be a better means of minimizing crosslinkage of the resin and thereby optimizing antibody access to the antigen. A full description of this method and rational can be found in my immunogold review article in the Journal of Histotechnology, vol 16, no 3, Sept 1993. Alastair McKinnon <a.d.mckinnon@abdn.ac.uk> 12 Mar 2007

Many thanks to everyone who replied, on or off-line, to my question about LR White polymerization. Most agreed that LR White polymerizes well at 50°C in 24 hours, and that sectioning and beam stability are good. There were, however, some interesting variations suggested. One respondent uses microwave polymerization. Another uses UV polymerization at 4°C. Another suggested that polymerization at 37°C for 3 days might reduce loss of antigenicity. Several people emphasized the importance of excluding oxygen and using gelatin capsules when using heat to polymerize, and one suggested degassing the resin prior to use. The most interesting suggestions involved using the "cold cure" method. The instructions that come with the LR White kit recommend not using this method for immunogold because the exothermic reaction can heat the resin above 60°C. But Dr. McKinnon (J. Histotechnology 1993: 16(3)) and others report superior results with the cold method. Apparently, if the resin can be kept cold during curing, the low temperature and shorter curing time reduce loss of antigenicity. A newer protocol using PTA during processing was also suggested. See Arch Histol Cytol 68 (5), 337-347 (2005). Ralph Common <rcommon@msu.edu> 13 Mar 2007

We routinely use 50°C for 24 hrs. No problem — must be polymerized in airtight containers though. We've only used gelatin capsules, but I think there is a PCR microfuge tube we tried a long time ago that also worked. There are commercial capsules that are more transparent for UV polymerization. Any LR White exposed to air (oxygen) will not polymerize, so even in the gelatin capsule, where the lid will have a small bubble, there will be a small amount of liquid to remove. The other method mentioned above — if the antigen appears to be sensitive to heating and no labeling occurs, you can try to "cold" polymerize by placing the material in capsules that are UV transparent. Place in a container with dry ice and a UV bulb. There are several commercial companies that produce these special beer coolers with fans and lights and reflectors for even polymerization. John Shields <jpshield@uga.edu> 15 Mar 2007

SPECIMEN PREPARATION - pollen grains

I am a post graduate student at the University presently studying the transport of pollen grains in dust. I have been using the Burkard 7 day Spore Sampler to trap the pollen grains and stain them for viewing under the light microscope. I am presently exploring taking electron micrographs of the pollen grains but this would require removing the grains from the greased Melinex tape of the sampler and mounting it onto the swab. I have come across the acetolysis process but this does not involve the removal of the pollen grain off the greased Melinex tape. Does anyone know of a technique that can be used to remove the pollen from the tape (by dissolving the tape perhaps?) so that they can be placed on the swab? Marissa Gowrie <marissagowrie@yahoo.com> 14 Mar 2007

I have to admit I'm a little surprised. When I was more active with pollen collecting, I found Ronald Kapp's acetolysis method to dissolve/attack just about everything accept the pollen exine wall! The problem is, after acetolysis, you can only compare those grains to reference grains which have been prepared the same way. When I examine air dried pollen from dust, I see that in many cases it's not a very good match to my acetolysis collection or to the key in Kapp's book. Still, it's better than nothing. Not being familiar with the system you are using I can't comment on solvents, but I would try solvent, followed by dehydrating agents to remove any water condensed from the evaporative cooling of the solvents and compare those samples to air dried reference samples. Frank Karl <frank.karl@degussa.com> 14 Mar 2007

SPECIMEN PREPARATION - tripod polishing problems

We have been trying to prepare a specific area cross-section using a tripod sectioning device. We have many years experience with the technique, but are perhaps a little rusty. We have been using fresh M-Bond to adhere a coverslip to the sample and then curing it for 1-2 hours in a Teflon vise in an oven or on a hotplate at approximately 150°C as usual. We have great difficulties with obtaining bubble-free adhesion (area where the M-bond is missing). Any help in making this technique more reliable will be very helpful to us. Sandra Keller <swtkeller@yahoo.com> 19 Feb 2007

I do a lot of epoxy work in thin sections although none of it is for TEM work. Have you tried putting your sample in a vacuum for 10-30 minutes? It doesn't have to be a very hard vacuum; something similar to the vacuum pulled by a gold sputter coater. Becky Holdford <r-holdford@ti.com> 19 Feb 2007

Ron Anderson told me that the IBM group deposits several microns of SiO_2 on their samples instead of using glass slides. Is it possible that you can find a colleague that can put a coating down for you? Ron Anderson always suggested that if you didn't have a SiO_2 coater, that you could configure an ion mill to sputter quartz onto the sample. It just takes longer. Another thing that you might try prior to putting the glass slide on with the epoxy is plasma cleaning your sample and glass slide. It is possible that epoxy is just not wetting the surfaces. The plasma cleaner will change the contact angle, hopefully for the better. Another question: By fresh, do you mean freshly purchased or freshly mixed. Even the unmixed M-bond stored in a refrigerator has a limited shelf life. Scott D. Walck <walck@southbaytech.com> 19 Feb 2007

SPECIMEN PREPARATION: ruthenium tetroxide staining recipe

Does anyone have a protocol for staining a 2 phase polymer film with ruthenium tetroxide? I need to try to stain a FIB cross section mounted on an Omniprobe grid. I'm thinking vapor staining, or possibly 'en bloc' staining of the film before I FIB it, it's about 30 nm thick on top of a silicon wafer. The finished cross section is roughly 50 nm or so sample thickness. We've already tried vapor staining with OsO4, it has no interaction with this particular system. Leslie Krupp <lkrupp@us.ibm.com> 19 Mar 2007

The recipe for preparing ruthenium tetroxide *in-situ*, as well as other important tips, follows. Important safety note: RuO_4 vapor is a very strong oxidizer. Therefore, all work involved in the preparation, use and disposal of RuO_4 must be done in a good exhaust hood (> 100 cfm exhaust). Safety glasses with side-shields (or goggles) and gloves (nitrile is best) are a must. To make the stain, weigh 0.02 g of ruthenium trichloride hydrate ($RuCl_3$-xH2O, CAS 14898-67-0) into a small (5 ml) vial. Add 1 ml of (NaOCl (10-13 %, CAS 7681-52-9) and agitate with a Pasteur pipet until the $RuCl_3$-xH2O has dissolved. The resulting solution should be deep reddish brown. Cap the vial immediately. I generally affix the sample to be stained to the inside of the vial cap and stain for the prescribed amount of time. The duration of staining for your polymer blend will have to be determined empirically. To safely dispose the stain following use, reduce by adding an excess of 10-15% aqueous sodium bisulfite ($NaHSO_3$, CAS 7631-90-5); the reduced solution will become pale green to blue overnight, at which time it can be properly disposed. All chemicals are available from Sigma-Aldrich and (presumably) other vendors. Although I recommend the NaOCl noted above, in a pinch, household bleach may be used instead of the reagent grade variety. The concentration of household bleach does decrease with shelf life so be sure to purchase a new jug with a long expiration date. If you plan to use this procedure over a period of time, purchase the good stuff and keep it refrigerated. The advantage of using 10-13% reagent-grade NaOCl is that a usable concentration of the NaOCl remains in solution over a longer period of time. Always refrigerate to prolong shelf-life. Prior to staining, one must first know the composition of the polymer blend and which phase is preferentially stained by RuO_4. You will have to empirically determine whether staining should be performed en bloc or following FIB milling. Several resources on RuO_4 staining of polymers are available to you: (1) A good reference for the selectivity of various stains is "Polymer Microscopy" by Sawyer and Grubbs (3rd ed.?). (2) Two papers on the subject are authored by Trent *et al.* and Montezinos and are referenced in the book. (3) I included an in-depth appendix on the details of stain preparation and use in G. M. Brown and J. H. Butler, New method for the characterization of domain morphology of polymer blends using ruthenium tetroxide staining and low voltage scanning electron microscopy (LVSEM), Polymer 38 (15), 3937 (1997). Gary M. Brown <gary.m.brown@exxonmobil.com> 20 Mar 2007

SPECIMEN PREPARATION – embedding mouse lens & eye

I would like a method to fix and Araldite/DDSA-embed a mouse eye for examination under a transmission electron microscope. The mouse would be approximately one-month of age. Would you please help me? Paul Toselli <paul@biochem.bumc.bu.edu> 20 Mar 2007

Do you really want to fix and embed the whole eye? Even assuming you could cut wrinkle-free thin sections of such a large block I don't think the section would fit on a grid. And, with such a large section, you would never find what you were looking for in the EM. I suggest defining your research target more precisely. Also consider collaborating with an anatomist/morphologist skilled in EM. If you want to look at the lens you should know that the lens is very difficult to infiltrate so Araldite/DDSA may not be the best choice of epoxy. Go to the library and get a good textbook of ophthalmology (or even your old histology text from medical school). The references will tell you what those who have already attacked this problem have done as far as fixation, embedding, *etc*. Why re-invent the wheel? Geoff McAuliffe <mcauliff@umdnj.edu> 22 Mar 2007

Whilst this is good advice from Geoff, fixing and processing the whole eye has its advantages in minimizing disruption to the internal structures, and in particular the retinal layers. Whole eye however, is extremely difficult to infiltrate and the great variation in structure density makes this a challenging proposition. The following procedure improved our LM sectioning results, and I would suggest giving it a try for EM processing. Following initial fixation at 4°C in 2.5% glutaraldehyde, trim a superficial slice off one side of the eye - just enough to expose the inner cavity. We use indented bluetack to hold the eye in position whilst slicing with a razor blade in a line parallel to the plane of the optic nerve which is pointing down into the bluetack. Don't be tempted to go for a second parallel slice as this causes a lot of disruption to the internal structures, and return the eye to fix for a further overnight period at 4°C. Follow this with a prolonged processing schedule using a low viscosity epoxy resin. If (safe) Spurr's is no longer available you could try TAAB's low viscosity epoxy resin. We have already tested this product as an alternative to Spurr for when our supplies run out. It is slightly more viscous than Spurr's but readily available. I would then suggest doing a final dissection to remove the lens (if not required) and to obtain blocks that are of a reasonable size for ultrathin sectioning and to include your area of interest. If this includes the retinal layer, it would probably be useful to include a bit of the optic nerve as an identifier. I would then suggest returning the samples to fresh resin for a final infiltration with pure resin (under vacuum at 50°C) for an hour or so, prior to transferring to embedding molds and polymerizing at 60°C for 24-48 hrs. Flat embedding will probably work best if it is a cross section you are going for. Alastair McKinnon <a.d.mckinnon@abdn.ac.uk> 22 Mar 2007

SPECIMEN PREPARATION - pre-embedding cells in agar

I have been given a protocol for embedding Cyanobacterium synechocystis from liquid culture, after secondary fixation, the cells are embedded in 2% agar prior to dehydration and resin embedding. When I have tried this method before, the agar has set so rapidly I have had insufficient time to incorporate the cell suspension evenly within the agar. Details of the type of agar, temperatures and method of incorporation are absent so any help on the practicalities of achieving a fairly uniform distribution of cells would be gratefully received. I usually work with the cell suspensions in Eppendorf (micro-centrifuge) tubes. Carol Evered <carol.evered@warwick.ac.uk> 29 Mar 2007

I have normally just embedded a spun down pellet in agar so the problem of dispersing is not normally an issue. I have read however, of a method that involves: 1. Melt 2% agar and then store in 50°C water bath, 2. Take a fixed and washed pellet (in Eppendorf tube) and warm in 50°C water bath, 3. Add warm agar to pellet and re-suspend, 4. Leave for 5 min in water bath, 5. Spin down rapidly 30 sec to 1 min — any longer and agar may set too soon. I am not sure if you want to keep yours dispersed though. 6. Cool in refrigerator or ice bath, 7. Chop up agar as required. The original method is: Hirsch JG & Fedorko ME (1968) Journal of Cell Biology 38:615. But is cited in a large double volume: Procedures in Electron Microscopy A.W. Robards & A.J. Wilson (editors) 1993 John Wiley ISBN 0 471 92853 4; pages 5:9.3-4. There are different melting point agars so it might be possible to experiment with temperatures or you could even try acrylamide gels which are cold setting. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 29 Mar 2007

You should try a low temperature gelling agarose. We use Sigma Type VII regularly. Keep the agarose at about 40°C until needed. Fix the cells as desired while in suspension. Use the agarose as the last step prior to dehydration so you are sure all cells are fully exposed to fix and washing. Spin the cells down in the Eppendorf tube and remove the supernatant. Then add ~0.5 ml agarose. Gently stir the cells up a little to get the agarose to enrobe them while keeping the tubes in warm water so the agarose remains liquid. Spin, and the cells should have plenty of time to pellet again before the agarose sets. Trick is to not try to resuspend the cells completely in the agarose or the ones at the top will not have time to get to the bottom before the agarose sets up. We then cool the tubes in ice (or very cool water) for a few

minutes. Inject water or low percentage ethanol toward the bottom of the tube by slipping the pipette down the side of the agarose plug. If you do this gently you can get the plug to release and float up so that it can be dumped out and then the pellet sliced into appropriate sized pieces for dehydration and infiltration. Debby Sherman <dsherman@purdue.edu> 29 Mar 2007

I have done this many times. I use low melting point agarose. Heat the Eppendorf tube (with the bugs in it) up to 37-40°C. Mix your bugs and agarose in the warm tube. Once the bugs are mixed (pipet up and down a few times) then put the sample on ice to cool the bugs and harden the agarose. If you need to, you can heat the tube more, which will give you more time to work until the agarose hardens. If you work fast and ice the sample immediately after working then the increased temperature has not caused me problems. I have used the same technique for many kinds of suspended cells. I have found this to work well. David Elliott <elliott@arizona.edu> 29 Mar 2007

Yet another protocol for agarose embedding is this one, that I got from the University of Bristol Veterinary Pathology website (http://www.bristol.ac.uk/vetpath/cpl/emtechs.htm): Prepare a 1.5% solution of agarose (Sigma Type VII is what I use) in distilled water by bringing to the boil while stirring. Spin samples at 5,000 rpm for 5 minutes. Decant supernatant from sample tubes and take them and the agar solution to the centrifuge. When the agar has cooled to ~60°C, quickly fill each tube with it, resuspend the samples (vortex briefly) and spin them at full speed for 30 seconds to 1 minute (I do a minute at 13,000 rpm). Do a maximum of 4 at a time or the agar will set before the sample can be spun down to the bottom of the tube. Cool the tubes by putting in a refrigerator or you can use a beaker of ice water. I remove the agar plug by cutting the Eppendorf tube side with a razor blade and pulling out the agar. However, this is not the safest procedure, and I like Debbie Sherman's suggestion about using water or ethanol to remove the plug. I then cut off the end containing the sample, and cut up the sample end into cubes. Jessica Cervantes <cervantes@bendres.com> 29 Mar 2007

We have also done this technique several times with great success using both low melting point agarose and sometimes using 12% gelatin. The concern with using agarose is the potential to introduce bubbles that sometimes don't move during centrifugation. Using gelatin is sometimes easier because of the lower viscosity, even at 12% and as long as you keep the gelatin below 30°C it will stay solid. We buy our gelatin from the supermarket; yes, the same stuff used for cooking and making jellies (we use Knox gelatin). This evolved from using gelatin for the Tokuyasu technique. Garnet Martens <gmartens@interchange.ubc.ca> 29 Mar 2007

Gelatin or agarose can be used to support cell suspensions for subsequent sectioning. One advantage of gelatin over agarose is that if the gel sets before the cells have been pelleted down, the gelatin can be easily liquefied by warming to 37°C. Agarose needs a little more heating to liquefy. One important point to remember is that if the cells have been fixed in aldehyde, residual aldehyde has to be either removed or quenched or it will cross-link gelatin or agarose before you are ready to let it gel. Wash the cells in a low concentration of ammonium chloride, lysine or glycine before embedding in the gel. Paul Webster <pwebster@hei.org> 29 Mar 2007

I have read with interest this thread on embedding cells in agar. The procedure I have used successfully for years is quite close to that described by Debby Sherman. As she and others have pointed out, its necessary to keep the melted agar, fixed and washed cell pellets, pipettes, tubes, *etc.* warm to prevent premature freezing of the agar, so it goes into the microfuge at about 40°C. I then spin down for 10 minutes at 14,000 rpm, a bit more than others have recommended, but I want to be sure that I get those puppies down! I'm not sure why you want to evenly disperse the cells into the agar. Usually a tight, enrobed pellet is

desired so that when you view sections you will see lots of cells fairly close together. But it is necessary to gently mix the cells into the agar just a little to effectively enrobe them but without diluting the pellet too much for the above reason. I mix my low melting point agarose (Sigma, # A9414) to 2% w/v, and keep my water bath for keeping the melted agar and cell pellets warm in 1.5 ml Eppendorf tubes at about 42°C. Between uses, I store the dissolved agarose stock in the freezer. The only other thing I would add to this discussion is to point out a paper by Jacqueline Wood and Karen Klomparens in which agarose, agar and gelatin were compared as encapsulating media for bacteria, yeast and mitochondria. They conclude that agarose has advantages over the other two; mainly that it contributes the least background density in the TEM image. As a result of reading this paper, I switched from agar to agarose. The reference is: Wood, J. I. and Klomparens, K. L. (1993) Characterization of agarose as an encapsulation medium for particulate specimens for transmission electron microscopy. Microscopy Research and Technique 25:267-275. Gib Ahlstrand <ahlst007@umn.edu> 29 Mar 2007

I would just like to add that we use 2% "ultra-low gelling agarose" Sigma Type IX. Mix in 2x strength buffer and use a volume equal to the cell suspension. This agarose melts at ~50°C but only gels at 8°C-17°C, allowing you to work with tissues at room temp and then gel when you are ready by placing on ice or in a refrigerator a few minutes. For very sensitive materials, working at ~25°C may be an advantage. In other respects it is handled just as detailed in all the other replies. I use a piece of sheet Teflon from Small Parts Inc. to work on and the agarose drops easily float off when set. Dale Callaham <dac@research.umass.edu> 29 Mar 2007

As I have mentioned in an earlier post, an alternative that is to encapsulate in alginate, we have used this to encapsulate both individual cells and tissues. The advantage is that you work at ambient temperature (no heating), disadvantage that you introduce calcium ions into the system which you may not want to do. One method we used was to use a 2% solution of sodium alginate and solidify by dropping into or flooding with 50 mM calcium chloride. I have used this with plant cell suspensions in the past after the primary fixation. Ian Hallett <ihallett@hortresearch.co.nz> 29 Mar 2007

SPECIMEN PREPARATION - annealing tantalum

I would like to anneal tantalum and I wonder if there is a big difference between "annealing tantalum under vacuum" and "annealing under argon atmosphere in a tube furnace". I have the second option available and I am not sure if there is still a chance of growing a thin oxide film, other than the native oxide thin film, on the top of the tantalum surface. Hany Ramadan <ramadanhany@gmail.com> 26 Mar 2007

At temperature, tantalum has a very high affinity for oxygen. The accepted practice is to anneal tantalum under vacuum. First, the tantalum must be clean. It should be degreased, and then can be chemically etched with a solution of 60% HNO_3, 20% HF and 20% H_2SO_4. After the tantalum is vacuum annealed, and the temperature has dropped below 1000°C, the chamber can be backfilled with 15 mm Hg high-purity (99.995%) argon. It must cool to below 200°C before removing from furnace. Stu Smalinskas <smalinskas@yahoo.com> 26 Mar 2007

IMMUNOCTYOCHEMISTRY – BSA purity for use as blocking agent

Bovine serum albumin (BSA) is often used as a blocking agent in immunoEM protocols (fraction V as I remember). However, there are many kinds of BSA products like: cold ethanol precipitated, heat-shocked processed, IgG free, globulin free, fatty acid free. Are there any differences between them that matter in immunolabeling? Or just buy the cheapest one and it will be OK? Aleksandr Mironov <aleksandr.mironov@manchester.ac.uk> 27 Mar 2007

Let me start by admitting I have not actually done a rigorous test of this, but I generally buy the IgG free version for all my immunocytochemistry work. I do a lot of immuno-staining of 0.5 µm semi-thick sections on glass slides and a fair amount of EM grids and a bottle lasts a long time. If I was doing several Western blots a day, I would be more concerned with cost. If you look at the specifications of many "high quality" BSA's, they are 97-99% pure. That is a great level of purity for many applications but the 1-3% impurities are generally immunoglobulins. IgG is 15% of the protein in human serum. If you are using secondary antibodies that don't cross react with bovine IgG, it might be unimportant. If you are using protein A or protein G, it could start to be significant. Tom Phillips <phillipst@missouri.edu> 27 Mar 2007

IMAGE ANALYSIS – phase analysis

I have a situation with an image analysis system attached to an optical metallograph and I would appreciate comments. We are evaluating thermal spray coating for area percent porosity. Our program has both single phase and multi-phase area percent analyses sub-routines. Keeping the region of interest (field) constant and the discrimination threshold also constant, the two phase analysis programs return different values (e.g., 3.2% vs. 5.5%). Multiple operators have experienced the same issue. Chris Holp <holpc@firstenergycorp.com> 15 Feb 2007

Offhand, it sounds like a thresholding issue. You should have some way to review the thresholded image to compare it to the original. Area measurements can be quite sensitive to the settings, and your results seem to indicate that your application is one of those sensitive cases. It does raise the question of confidence in the answer when the results vary so widely. What are the differences between single and multiple phase modes of operation? Offhand, I think you would want single-phase mode, but multi-phase mode should collapse to single-phase mode and give you the same answer if the same threshold was used. If your system automatically chooses a threshold, I would be very wary of the results. I prefer operator setting and review to make sure you are measuring what you want to. Warren Straszheim <wesaia@iastate.edu> 15 Feb 2007

At first glance 3.2 relative to 5.5 is a huge difference, but digital image thresholding also implies these conjugate values, 96.8 and 94.5, which are not so different. The small difference between the two softwares might reflect their different philosophies regarding how to treat the pixels at the edge of the ROI and/or image frame. The thresholding may also have some differences. For example, one might include in the count only those pixel values greater than the threshold value, while the other includes what is greater OR equal to the threshold. You may be able to figure out what is going on by downsizing the same image to fewer and fewer pixels. At some point I would believe both softwares would give you the same answer and thereby provide clues as to what's happening. Michael Shaffer <michael@shaffer.net> 15 Feb 2007

We have jointly run evaluations paying particular attention to the field area as well as manually setting the gray scale thresholding value the same, analysis to analysis. The multi-phase mode will take up to five thresholding ranges to return area percents for each thresholded gray level. But as you suggest, Warren, the multi-phase mode does not require more than one threshold range be defined. We did run an experiment with a sample where we were able to establish two gray levels diagonally in an image (black on one side, white on the other). Our field of interest box was set so that the two levels ran corner to corner to establish approximately 50% area. While the answers were closer then, there was still some difference between the two modes. Chris Holp <holpc@firstenergycorp.com> 15 Feb 2007

PHOTOGRAPHY – neutral density filters

I would like to address the collective wisdom on neutral density

filters. I'm using Hoya camera ND filters to lower light levels for photomicroscopy while maintaining color temperature. But I've convinced myself that these filters have a slight greenish cast. Is this just my fancy running away with me? Who makes a good grade of ND filter? I don't want to use the Kodak gel filters as I prefer the weight and strength of glass. Any suggestions? Frank Karl <frank.karl@degussa.com> 15 Feb 2007

If they are true ND filters made correctly by a reliable manufacturer there will be no greenish cast. Tiffen, Hoya, Nikon, etc., should all be free of color casts. I can't vouch for "Acme Filter Co." or "Joe's Filters". That said, there may be issues of older filters having developed an off-color as dyes or plastics age. I am not extremely familiar with the various methods of making filters, but I strongly suspect that some methods are more stable than others over geological time. Randy Tindall <tindallr@missouri.edu> 15 Feb 2007

I agree with Randy Tindall that 'good quality' NDF's are neutral in the visible. But if yours has gone green for whatever reason, you can make nice ones yourself by evaporating aluminum foil on glass. Of course, you have to be careful of the surface, but by changing the time of evaporation (and/or distance to the source) you can get nice NDF's pretty cheap (assuming you have an evaporator). Tobias Baskin <baskin@bio.umass.edu> 15 Feb 2007

Assuming we are talking about digital cameras, there is a wonderful little freeware program that will allow you to correct for problems with illumination color, uneven illumination, and the color response of your camera sensor. The program is "Image Arithmetic" and can be downloaded from http://www.t3i.nl/myblog/?page_id=7. The feature of interest is image division. The subject image is divided, pixel by pixel, by a blank image taken under identical conditions of illumination. You must do this in manual mode to keep the illumination intensity identical. Obviously the extra steps will slow things down, but the procedure works very well when done correctly. Ralph Common<rcommon@msu.edu> 15 Feb 2007

DARKROOM - uneven printing

I have printed EM micrographs for years but am having a problem that I've never encountered and wonder if anyone else has had this same problem and resolved it. On very high contrast negatives I have no problem. They print just like normal and look wonderful. No background unevenness and they look fine. On negatives with low contrast, however, those requiring F4 and above filters, have a very uneven background and the image looks streaked. Clear areas over vacuoles have grey blobs that are not present on the negative. At first I thought it was the filter sets and bought a new set of Ilford contrast filters. Same problem. My print paper is older but stored at 4°C and I've never had a problem keeping older paper around like this. Andrew Bowling <abowling@msa-stoneville.ars.usda.gov> 16 Feb 2007

It sounds to me like you may have unclean, smudged surfaces somewhere in the optics of the enlarger. The fact that the background is uneven may mean that your optics are out of alignment. A few places to check: 1. If you are using glass negative holders to keep the TEM negatives flat in the enlarger, there are 4 surfaces there to check (2 each, top & bottom glass) and clean if you see smudges or dirt. As they get handled all the time when printing, they are vulnerable to finger grease smudges and dust. And as those surfaces lie close to the negative, any "detail" on those surfaces may well lie within the depth-of-focus of your projection lens, especially when the lens is stopped down as is usual when printing, and get superposed onto your negative's image and thus wind up in the printed image. The use of higher contrast filters would greatly enhance those smudgy artifacts and uneven illumination or background, which may have been there all along but not noticeable when using normal contrast or no contrast filters, especially when present in the typical "busy" image of a stained section, or negative stained sample. 2. Check the projection lens surfaces for smudges. 3. Inspect the top or bottom surfaces of the condenser lens stack, especially the bottom one as its positioned right above your negative, so any "detail" on that

https://doi.org/10.1017/S1551929500055607 Published online by Cambridge University Press

surface may also lie within the depth-of-focus of the projection lens as I explained above in #1. One way to test for such smudges and dirt on the optics, is to remove the negative, stop down to smallest aperture to increase depth of focus to better reveal any contaminants on glass surfaces near the negative, and of course to reduce intensity of light on paper which will be high with no neg inserted, and put in the #4 or #5 contrast filter. Do a test exposure series to get roughly mid-tone gray or slightly denser, and then expose and develop an 8x10 print. Ideally, you would expect a uniform gray tone all across the 8x10 print, though one usually sees a bit of fall-off around the edges. However, if any of the surfaces mentioned above are dirty, you will see their patterns in the test print. Then it is just a matter of cleaning surfaces and performing more exposure tests until the imperfections have been removed of reduced to an acceptable level. I used to do this kind of test periodically when I used an enlarger, and though I no longer use one having gone digital - sigh! - these principles still apply to keeping flatbed scanners free of imaging artifacts due to dust or smudges on their glass surfaces. Gilbert Ahlstrand <ahlst007@umn.edu> 16 Feb 2007

A couple of chemical questions: is your paper developer fresh? And do you use an acid stop bath? I have seen this sort of thing with developer nearing exhaustion. I have also seen it with fresh developer when I once had to make 16x20 prints in a very limited space. I had zero room for more than two trays and tried putting my developed prints directly into fixer (I couldn't even leave the room to do a water rinse, since it was the only dark place around and the toilet bowl was too small, but that's another story...). No matter how long I left the prints in fix before checking them with the lights on, some of them would mottle and streak. Along these same lines, if you have any sort of uneven developing in your film processing this will jump out at you when using higher contrast filters or paper to compensate for a thin or low-contrast negative. If, as Gib rightly suggests, you have dirt in your enlarger's optical path it will show up and be amplified by the high contrast filters. If so, the pattern of blobs and streaks should be similar from print to print. If the pattern changes a lot, I would suspect film agitation as the culprit. This can even happen in nitrogen burst systems, not to mention the good old lift and tilt method with the TEM film racks. We use a combination of both, since a few plugged holes in the nitrogen tubes can set up repeating patterns in your developer, leading to streaks. In a high contrast negative, you would probably never notice this, but it would jump right out at you in a low contrast neg. I would do the tests Gib suggests with paper, along with doing a few blank exposures of EM film to get an even, featureless grey on the film. If it really comes out even and featureless, your film processing is probably good, but if your film looks blotchy and streaky, look no further before modifying your agitation technique. Randy Tindall <tindallr@missouri.edu> 16 Feb 2007

One other thing that might be worth trying would be to simply fog a sheet of paper with light rather than the enlarger. Experiment with strips of paper and a distant low wattage light until you get the right sort of grey. This might help to eliminate the paper as the faulty item. If you wanted to test a particular contrast filter you could hold it in front of the light as well. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 19 Feb 2007

CAMERAS - CMOS versus CCD

What are the advantages/disadvantages of CMOS compared to CCD cameras? Are they comparable in terms of sensitivity, resolution and durability? John J. Bozzola <bozzola@siu.edu> 08 Mar 2007

Both CMOS and CCD sensors convert light into electrical signals that can be understood and interpreted by a computer, and they both use the same physical effects for the conversion, so the underlying physical limitations are the same. The difference is the technology used: In a CCD chip, all the collected information in the many pixels of the chip are processed by a very limited number of electronic elements (voltage amplifier, *etc.*), often only one. While that can be a drawback in terms of speed, it does help with the uniformity of the signal. Plus, the individual pixels of the chip can be almost entirely used for converting photons to electrons, *i.e.*, the CCD chips use almost 100% of the available area as light sensitive areas. For even higher sensitivity one can thin down the chips and illuminate them from the back side. A CMOS (Complementary Metal-Oxide Semiconductor) chip uses a different technology for the fabrication. It is actually the same process that is used for regular electronics, so it is easy to also integrate some electronic devices on the chip. In a CMOS chip, each pixel has its own voltage amplifier and perhaps other electronics. In comparison to a CCD chip then, the uniformity is usually worse (many amplifiers as opposed to one), and the sensitivity is not as good (some "dead area" for each pixel). On the other hand, CMOS chips can integrate other electronics on the same chip ("camera on a chip"), and they can be much more energy efficient (hence their use in consumer type cameras that depend on batteries). The fact that the electronic circuits on each pixel eat up some space made CMOS quite useless for light sensitive chips until a few years ago, when the dimensions of those electronic circuits had shrunk to a size that allowed a significant area of each pixel to be used as light sensitive area. Factors like resolution are not quite as simple as they appear. You can make cameras with really small pixels, but what happens is that the number of electrons you can store in each pixel becomes smaller and smaller. Comparing this number to the spontaneous generation of electrons (noise), you can see that the dynamic range of the camera gets reduced. For example, if you can store 100,000 electrons, and your noise level is 50 electrons, you can distinguish 2000 intensity levels, or about 11 bits. If your storage size shrinks to 10,000 electrons, you end up with 200 levels, or less than 8 bit. On the other hand, if you increase the pixel size, it takes longer to read them out, and the chip gets a lot bigger (=$$). But this applies to both CMOS and CCD. So, for any given set of parameters there is a different chip that is best. That could be a CMOS or CCD chip. My personal opinion is that we will see more development in the CMOS sector (due to their use in consumer cameras), and they will start to eat into the CCD market. But I don't think that the CCD market will totally go away, especially in the scientific arena, where it is often necessary to squeeze some signal out of the last photon. As far as durability goes, I don't see a reason why a CMOS chip should have a durability that is different from a CCD chip. If you need more information, just google "CMOS CCD", and you'll get a lot of information that goes deeper than what I wrote up here. Michael Bode <mike.bode@olympus-sis.com> 08 Mar 2007

As usual, it depends. Here is a link to a good comparison: http://www.dalsa.com/markets/ccd_vs_cmos.asp The uniformity of CCD imaging is a big deal, as well as lower noise (which the link does not really address). This link: http://www.imaging-resource.com/PRODS/D30/D30A4.HTM does discuss noise. Both links discuss the added external circuitry needed to read out the analog data from CCD. But having on-chip A/D increases chip size and also puts pressure on being able to reduce pixel dimensions. The recent availability of smaller feature size CMOS helps in this respect. It also helps with off-chip signal processing which both sensors have to have. The choice depends on application and target price. For imaging satellites, CCD is the choice. So too for high quality telescope cameras (Peltier cooled). To my knowledge, no one has made a cooled CMOS sensor. CMOS has inherently more shot and thermal noise than CCD. This does not really affect the consumer market. But for professional users, their cameras use CCD. The use of CMOS by Canon in an SLR is a first and will be interesting to follow. Gary Gaugler <gary@gaugler.com> 08 Mar 2007

MICROSCOPY - analysis of paper

Do you have an idea which microscopic technique would be suitable for analysis of paper? For 3D, I did trials with confocal laser scanning microscopy, which were not truly successful as grey scale differences were too low. Other techniques I know would require splitting or sectioning, as far as I know. Do you have further experience? For 2D, I tried light microscopy and SEM, but grey scale differences were to low here too in order to differentiate between components. Could TEM be useful? What could help to get better distinguishable features? Greta Rennings <greta.rennings@web.de> 07 Mar 2007

If you what you mean by "analysis of paper" is a full 3D structure, the best I have ever seen was done by a company offering a proprietary service where serial sections are imaged on the block face. I have no commercial interest and am not endorsing them in any way. http://www.microsciencegroup.com/applications_publications.htm This page links to their applications papers. Select the one called "Filtration + Separation" for a Sept, 2001 publication showing an example of filter paper. [I was surprised to see they also had a link to a Newsweek story in which I was interviewed.] I, and many others, have had various degrees of success cutting and registering serial microtome sections of embedded paper. My preferred current method is SEM imaging after cross sections are prepared by embedding, polishing, and etching. It is illustrated in my abstract in the 2002 MSA Annual Meeting, Page 178. A key reference for the method is G.J. Williams and J.G. Drummond, J. Pulp and Paper Science, V26 (2000), P. 188 Final note. Surface structure is very well characterized by some of the modern white light interferometers. No preparation needed. David R. Rothbard <rothbardd@netscape.net> 08 Mar 2007

Paper is one of my favorite specimens (the other two are insects and glass) for the initial demonstration of SEM capabilities to students. It always has a beautiful structure whether coated or non-coated (observed in low voltage or environmental mode), and it is very easy to handle. So, I do not understand why for you "grey scale differences were to low". If you can send me your images off-line we could discuss them in more detail. Vladimir Dusevich <dusevichv@umkc.edu> 12 Mar 2007

LM - Koehler illumination

Some books say that this must be performed on both low and high power as you focus on an object to be imaged (digital image). Others say that you only need to perform the Koehler steps for high power images. Do those who say to do it on both low, then high power, is that to 'get you in the ballpark' prior to going to high, or is this step unnecessary? Marilyn LeMieux <marilyn.lemieux@genzyme.com> 04 Apr 2007

Performing a Koehler alignment takes less than 1 min, so why bother about not doing it at lower magnification if this can help at higher magnification? If you think this does not help, why do you care? The purpose of it is to focus the beam on your object, in the condition you take the picture. That said, if you take all your pictures at the same high magnification, you probably don't need to perform a Koehler each and every time. Stephane Nizets <nizets2@yahoo.com> 04 Apr 2007

To quote: "The Koehler technique is recommended by all manufacturers of modern laboratory microscopes because it can produce specimen illumination that is uniformly bright and free from glare, thus allowing the user to realize the microscope's full potential." Find out more at: http://micro.magnet.fsu.edu/primer/anatomy/kohler.html and http://www.aecom.yu.edu/aif/instructions/koehler/koehler.htm. Note that you should check Koehler illumination every-time you change objective on a microscope, and setting Koehler illumination is crucial if you are using Phase Contrast (or DIC) optical contrast enhancement. So even low power phase objectives require Koehler adjustment for good images via transmission illumination. It is also required if you are capturing transmission images via a camera (or they will not look that good at all). For heavily stained sections at low

https://doi.org/10.1017/S1551929500055607 Published online by Cambridge University Press

magnifications you can get by without bothering, but as Stephane points out it takes very little time to setup and it is poor science not to check it every time you use the microscope (particularly as you will have spent many hours preparing the specimen). Previous users may have setup the optics incorrectly for various reasons. Koehler illumination is irrelevant with epi-fluorescent imaging as the light is backscattered into the objective, although often you will also want a standard phase contrast or DIC transmission image as well. Koehler illumination is essential for transmission images of unstained specimens with limited contrast (where phase contrast or DIC optics is often also required to enhance the specimens contrast by optical interference within structures inside the specimen). Poorly adjusted optics lead to very uneven illumination and the appearance of dark shadows in the image. It will very badly affect contrast enhancement optics (you won't get much enhancement). These problems are naturally best avoided, particularly as setting the optics correctly is so easy. All microscope manuals will tell you how to set up Kohler illumination with the microscope (plus other important things like aligning illumination bulbs and phase contrast rings). Expensive modern motorized microscopes can do much of this automatically these days. Keith Morris <keith.morris@ucl.ac.uk> 04 Apr 2007

If you drive a car with a manual gear shift, when you want to go, you push in the clutch, put it into first gear and let out the clutch; then you push in the clutch to put it into second gear and let out the clutch, then you push in the clutch and put it into third gear and let out the clutch. When you are driving a manual microscope, you click in the low power objective, change the diaphragm and adjust the condenser; when you want middle magnification (second gear) you change the diaphragm and adjust the condenser; and when you want high power, *etc*. I can continue to play with this analogy. I do not know why people can accept moving the focus adjustment on a microscope but not the condenser adjustment when magnification is changed. Perhaps these poor souls did not do well in optics when they took college physics. Bob Blystone <rblyston@trinity.edu> 04 Apr 2007

It is always good to "Koehler" every time you change lenses, especially if you are going to take pictures (digital or otherwise). "Koehler" aligns the illumination system with the rest of the microscope's optical axis, ensuring even illumination without odd shadings or shadows. Once you get the hang of it, it only takes a few seconds to do it, so it is certainly worth the effort. Leona Cohen-Gould <lcgould@med.cornell.edu> 04 Apr 2007

I can't stress strongly enough the importance of establishing Koehler illumination for all techniques. I agree strongly with several of the responses that stress how easy and quick the process is, once you have done it a few times. Koehler illumination establishes the "baseline" for all other imaging. Setting aside alignment of the lamp filament (which typically only needs to be done when the lamp is changed), it involves the simple setting of focus and apertures for three key lens sets: objective, condenser, and eyepieces. On most microscopes, each of these lenses has adjustment for focus. Also, it is important to understand the appropriate setting for the field iris (which controls scatter and glare) and aperture iris (which controls coherence and has a major impact on edge fidelity as well as resolution). Unfortunately, today's schedule doesn't permit a long discussion, but for those of you who are interested in a brief anatomy and physiology less regarding each of the three key lenses and their apertures plus a short recipe for establishing Koehler, send me an email with "Koehler, please" in the subject line and I'll try to send you a PDF early next week, when I am back in the office. The take away message: Please take a few minutes to become familiar with Koehler illumination and use it daily and check it whenever you move from one magnification to another or one technique to another. Your microscopy will improve dramatically. Barbara Foster <bfoster@mme1.com> 04 Apr 2007

Now, lets get one thing straight from the beginning. I use Koehler illumination. I think Koehler illumination is the mark of the competent microscopist. Don't use Koehler illumination? As one of friends says "Dude, that's just wrong!" But in truth the only scope I have true Koehler illumination is a monocular petrographic scope with a detached but focusable AO lamp with an iris. This scope is my own at home in my lab. All the scopes I have seen and used in the last 20 years were missing some feature which prevented true Koehler illumination. Some were lacking centerable lamps, others immovable ground glass filters while other did not have centerable objectives. Those that did had wire filaments and not ribbon filaments. I don't care whose brand. It seems impossible to set up true classic Koehler illumination. I don't even want to talk about focusable and centerable Bertrand lens! Frank Karl <frank.karl@degussa.com> 04 Apr 2007

LM - calibration standard Z direction

I am looking for a Z direction calibration standard for an optical microscope which can do surface profiles, magnification ranges from 20 to 1000x. Javaid Qazi <javaidqazi@kemet.com> 08 Mar 2007

The method I know of for calibrating the fine focus knob of the optical microscope is to measure the thickness of a microscope glass slide with a fine micrometer, put a mark with felt pen on both sides of the slide, offset from each other a bit, then record the fine focus reading for the focus on one mark and the fine focus reading for the other mark. Do this for each objective. The calibration is then based on the micrometer you use. Hope this helps. Regards, Mary Mager <mager@interchange.ubc.ca> 09 Mar 2007

Are you taking into account index of refraction of the glass, while measuring fine focus of the marks on both sides of the slide? My guess is that accuracy of such calibration will depend not only on the micrometer, but also on how accurately the index of refraction is known; it should be close to 1.2 - 1.5 but probably will vary depending on the source of the glass, *etc*. You probably could use height standards made for AFM, some even NIST traceable, there are many sources, just Google. Of course, the accuracy of calibration will still depend on the operator. Valery <vray@partbeamsystech.com> 10 Mar 2007

EM - field sources

I'm looking for some help in understanding electro-magnetic fields, and the sources generating them. Here's the issue in my lab we've gone from 0.46 mG to 8.7 mG in X-axis EMF-AC, and 1.1 mG to 15.2 mG in Z-axis EMF-AC. These obviously push beyond specs for the SEM in the room. So I am looking for an education. I have no idea of the context to put this in. What would or could cause these kinds of EM Field increases (15 to 20 times)? A single new 110V outlet? A new 1000W UPS in a near by room? A 480V feeder line? A new HVAC blower motor? These measurements were made in the middle of a 10 ft x 10 ft x 9 ft room (3.2 m x 3.2 m x 3 m) Next to an SEM, in the Off state (no power). I operate the rooms on two sides and below, and I have checked the labs on the other lateral sides and the floor above - no new equipment or significant electrical services within at least 15 to 50 feet in these spaces. But "significant" what is significant? Am I looking for a new 110v outlet? Or a computer UPS? Or am I looking for something in the next building? Or 150 feet down the hall? I have not dug through the ceilings yet to look for any "hidden" changes but again I do not know what I should be looking for. Am I looking for an induced current coming through an Ethernet cable? I do not have a hand held Gauss-meter to track things down. Do I need one? Any help would be great. Richard E. Edelmann <edelmare@muohio.edu> 07 Mar 2007

The changes that are observed won't be coming thru the Ethernet cable because this is low voltage/low current (tiny wires couldn't handle

https://doi.org/10.1017/S1551929500055607 Published online by Cambridge University Press

much, anyway). Single new 110 V outlet: no, but what is connected to it is important. Things with big motors (fridge/AC) or high voltage/current transformers are bad. Could be very bad. A new 1000 W UPS in a near by room? Probably not, but it depends on how close it is. Try moving it or turning it off to see if there are changes in the field you measure. This could be a contributor to the overall problem, but may not be the main source. A 480V feeder line? A definite possibility. Particularly, if there are transformers nearby. A new HVAC blower motor? A definite possibility, and a probable cause if it is a "big" one, and it is within 150 ft in any direction. Look for substations that service whole buildings (or more) The bigger they are, the farther away they need to be from your instruments - big ones may need to be 500 ft or more away. Also, newly installed or changed elevator motors are notorious offenders. Again, these may need to be hundreds of feet away. The vibrations are bad, too. In general look for transformers or electric motors in any direction (including up/down). The larger the voltage & current that go through them, the larger the EM fields they produce both in area coverage and magnitude. Paul-James Jones <pjones@rdg.boehringer-ingelheim.com> 08 Mar 2007

We've had to fight field issues in an old building. We've found that most of our fields can be accounted for by someone making a ground-neutral bond, *i.e.* tying a neutral to a ground line. This, while it is low voltage, can generate large currents and hence large fields. Neutral-ground bonds are against the electrical code but they still occur. I can recommend a hand-held Gauss-meter which is good to 0.01mG. Henk Colijn <colijn.1@osu.edu> 08 Mar 2007

TEM - cleaning Pt apertures

Does anyone have any experience cleaning the carbonaceous gunk off of Mo or Pt-Ir apertures with a solvent? I know about heating up in a Mo boat, but looking for alternatives. Will ammonium hydroxide do the trick? Or something else? John Fournelle <johnf@geology.wisc.edu> 07 Feb 2007

I've had very good luck for the last 30 years or so cleaning apertures with a cut knap polishing cloth and 1 micron diamond paste. Just place the aperture on the cloth with a little paste and put your finger on it and rub it in a circular motion. Do both sides. Clean ultrasonically in Joy dishwashing liquid and hot water, rinse in hot tap water or distilled water and immediately blow dry with a duster to avoid water spots. My understanding about Joy is that the Proctor & Gamble labs use it for cleaning critical parts of AAUs and can find no residue. Apparently this is not true of all dishwashing liquids. This works with 1 mil foil (including multi-hole strips), 5 mil countersunk and even the little Siemems apertures that are heavily countersunk. Just don't try it with gold foil self-cleaning apertures. The gold foil is far too thin and fragile for this technique. Chuck Garber at SPI tells me that most metallographic diamond pastes have silicones in them. That's a problem, but his pastes don't. As you are probably aware, heating moly in a platinum boat is a problem and even heating Pt has limited usefulness because the Pt recrystalizes and eventually you have an aperture with alligator skin and it won't work any more (high astigmatism). Polishing a ruined Pt aperture will restore it, probably due to the smearing of the metal by the diamond particles. All in all it's pretty good because you don't need to know what the aperture is made of, there's no complicated or exotic equipment needed (beyond an ultrasonic cleaner), no organic solvents or other nasty stuff, and you can use the same apertures for years. Once in a great while I might fold a 1 mil aperture, but that doesn't happen very often. Ken Converse <kenconverse@qualityimages.biz> 08 Feb 2007

Neat stuff. Will it work for very small Pt and Mo apertures - say a 10 µm objective aperture in a TEM? Sounds like a big time saver. Vitaly Feingold <vitalylazar@att.net> 09 Feb 2007

I don't think I've actually cleaned anything smaller than about 50 µm, but I also don't see any particular reason why 1 µm diamond shouldn't work on a 10 µm aperture. Give it a try. Ken Converse <kenconverse@qualityimages.biz> 14 Feb 2007

A 1 µm abrasive will leave 0.3 to 0.1 µm scratches. These will be visible at the edges. Jim Quinn <jquinn@www.matscieng.sunysb.edu> 14 Feb 2007

True, but does it affect the imaging? My experience with SEMs says no. I'm not sure about TEMs. Ken Converse <kenconverse@qualityimages.biz> 14 Feb 2007

I have used diamond paste to clean both SEM and TEM apertures in emergencies. Standard W filament SEMs have been fine and I have got away with it in a LaB_6 filament, 200kV TEM, 50 µm objective aperture (maximum magnification 330K). But I would not use it to clean apertures in a high resolution TEM objective; neither would I use it to clean condenser apertures in a FEG or for small probe modes (apertures of 20 µm or less). These are quite unforgiving and need good apertures. I would not want to break the vacuum again for the sake of buying a new aperture or cleaning one properly. 'Properly' is the subject of other discussions. Ron Doole <ron.doole@materials.ox.ac.uk> 15 Feb 2007

This is pretty much what I do. I thought I was missing a simple and faster method. But all my intuition screams for either brand new or a heat-cleaned small objective aperture for TEM. Will try diamond paste one day, when I have no other choice. Vitaly Feingold <vitalylazar@att.net> 16 Feb 2007

In the 1960s, we had two JEM-6A TEMs that used Pt apertures. The method we used for cleaning these apertures was based on the method we used in the analytical chemistry laboratory for cleaning platinum crucibles: namely, treatment with fused potassium bisulfate ($KHSO_4$). We placed the aperture in the bottom of a small ceramic crucible, covered it with a small amount of the potassium bisulfate, heated the crucible with a micro-Bunsen burner until the $KHSO_4$ melted, let it cook for a few minutes, and then let the $KHSO_4$ solidify and cool. Then we dissolved off the bisulfate with hot water, picked the aperture out and sonicated it in hot distilled water for several minutes several times, then sonicated it in isopropyl alcohol. The apertures came out bright and shiny, and never gave any trouble with distorted holes or evidence of any non-conducting stuff in the holes. I also did a bit of searching on the Internet, and came up with the following methods recommended by the British Crystallographic Association Industrial Group, for cleaning platinum laboratory ware: 1. Sonicate in 10% citric acid at 50°C. 2. Sonicate in 20% citric acid at 80°C. 3. Sonicate in 10% hydrochloric acid. 4. Treat with boiling chlorine-free nitric acid. Wilbur C. Bigelow <bigelow@engin.umich.edu> 20 Feb 2007

TEM - free lens control

Having struggled with my 'new' microscope for a few years now I am reaching the limits of my knowledge. I am looking at relatively large GaAs devices (>100 µm in diameter) and need to be able to take diffraction contrast images of the whole thing. The JEOL 2011 is great at magnifications >100,000x but if I try to get an image at 100x all I see is a tiny bright spot corresponding to the objective aperture. I suspect this means that the objective aperture is nowhere near the back focal plane of the objective lens in low magnification mode. Now, I know the kind of image I want was easy to get on my 1979 vintage 120CX, with a 2-stage condenser and one objective lens, whereas this beast has a three stage condenser plus a condenser and objective mini-lenses. This morning I managed to get a reasonable low magnification diffraction contrast image by playing with the free lens controls, (essentially turning off some lenses so it behaved more like my old machine). My question is: has anyone done this in a more systematic manner and could give me some directions on

which lenses to vary to get what I want? I could just about work it out myself with 3 lenses but I have no idea when there are 5. Not to mention 2 more in the gun, 3 intermediates and a projector, plus alignment lenses. Richard Beanland <richard.beanland@bookham.com> 21 Feb 2007

I am not familiar with the 2011, but in the scopes that I do know about, the functions of the objective and SA apertures are reversed in low magnification mode. If the objective lens current is very small in low magnification, then the beam goes through the specimen and is not significantly focused at the back focal plane, so that part of the beam that goes through the objective aperture has essentially been transmitted from the small portion of the specimen that directly overlies the aperture. The SA aperture is in a diffraction plane in low magnification, so it is a contrast-producing aperture; therefore, one can position it to get a contribution to the image from those diffraction spots that pass through; *i.e.*, one can select the spots to be included by choosing the appropriate size and position of the aperture, like DF imaging with the objective aperture at high magnification. Unless the 2011's optics are much different, this should not depend on how many lenses there are in the imaging system. Bill Tivol <tivol@caltech.edu> 21 Feb 2007

I have not used the JEOL 2011, but it is a TEM so it must follow standard TEM principles. Instruments usually switch off the objective lens to achieve very low magnifications. They use the diffraction lens to focus and gain some contrast through the inclusion of the intermediate or diffraction aperture. Try switching off the objective (some drop to 20% rather then switch off) and use the diffraction lens to focus. Balance the remaining lenses to reduce distortion if this arrangement causes problems. Introduce the diffraction aperture once you have a reasonable image. The image quality will not be good, probably in excess of 3 nm resolution, due to the very long focal length required. Steve Chapman <protrain@emcourses.com> 21 Feb 2007

I do seem to remember knowing at one time that the objective and selected area apertures did opposite things when the objective lens is turned off, but it has been a while. I did find I can get strong contrast in low magnification (LM) mode using the SA aperture and have an acceptable field of view, although I couldn't get dislocations to be visible at 1000x in LM mode when they are blindingly obvious in standard mode at 2000x. Also, there is a very significant increase in brightness if I turn C1 down below its normal minimum using free lens control. Unfortunately, to see the dislocations I have to have a small aperture in the diffraction plane, so it's no use using larger apertures. So some more experimentation is needed. If I do get a good setup using free lens control, I'm happy to pass the information on to anyone else who finds they have the same problem. Richard Beanland <richard.beanland@bookham.com> 01 Mar 2007

SEM - takeoff angle

We have a JEOL JSM 5310 LV scanning microscope (low vacuum, environmental type) with an Oxford Instruments EDS system and the LINK ISIS 300 software. I am trying to use SEM Quant for full quantitative analysis of minerals using a set of well characterized standards. I have done this before on a regular SEM very successfully, with the routine involving setting the working distance at 39 mm. However, with this LV SEM model, I have noticed that I am getting a very weak signal (very low number of counts) on my calibration standard (I'm using cobalt), but that the signal is appropriate if the working distance is reduced to 26 mm. My question therefore is: How critical is it to stick with a working distance (WD) of 39 mm for quantitative analysis and subsequent ZAF corrections? If I change the WD, will I need to make any changes to my protocol for quantitative analysis? If the WD is very critical, is there a way to improve the count rate while still working at 39 mm? Aley El-Shazly <elshazly@marshall.edu> 14 Feb 2007

First of all, although the instrument that you used in the past was optimized for EDS at 39 mm most modern instruments work with a far

https://doi.org/10.1017/S1551929500055607 Published online by Cambridge University Press

shorter WD. A simple way to determine the best WD is to place a stub in the instrument at a set magnification. Look at and record the count rate. Move the specimen Z to a new position at the same magnification and refocus before checking the new count rate. Repeat until the optimum, maximum count, Z position is determined, note the focal current or working distance for future reference. When you carry out an analysis, once this focal current or WD has been set, only adjust focus from area to area through a change in sample height in the stage - "Z". You will need to feed the new take off angle and azimuth (if any) into the software so that the corrections apply to the new position. From my notes on the operation of the 5300 with clients I see a working distance of 15 mm seems to be ideal! Steve Chapman <protrain@emcourses.com> 15 Feb 2007

Assuming your system was set up properly: After opening the ISIS application select the Dewar icon, then 'detector' then 'orientation'. This should list the detector conditions including the working distance. Ron Doole <ron.doole@materials.ox.ac.uk> 15 Feb 2007

Other replies have pointed out that the geometry for analysis varies between microscopes. We are still running a JEOL 840A that is setup for EDX at a working distance of 39 mm. Our Hitachi 2460N uses 25 mm, and we are hoping to get a new SEM that uses something between 5 and 10 mm. If your ISIS system was installed by the Oxford personnel then the proper geometric constants, including working distance, should have already been set up. You should have a Detector icon on the Labbook task manager. Start it, and then choose the Detector menu and the Orientation option. That should show you the preferred working distance for your microscope. You probably still want to perform the exercise of confirming the working distance for your maximum count rate. However, if the collimator on your x-ray detector has a cut out on the bottom, it is possible that you will get an optimum count rate at some slightly greater working distance. That would not be good as you want to be at the specified working distance. I have also found that indicated working distance is not the same for all voltages. I can set our Hitachi objective lens to a nominal 25 mm focal length and find that I have to raise or lower the stage as I go to different accelerating voltages to bring the image back into focus. You will need to determine the proper indicated working distance at a given kV for your actual desired working distance. One other thing - you need to make sure that the parameters stored with your spectra reflect your actual conditions and geometry. Many years ago, I collected several weeks of data on a Kevex system before I realized that we had not setup the software to store the correct conditions. Fortunately, we were consistent in our data collection and were able to correct the files, but it is better to get it right the first time. We have an Oxford ISIS on our Hitachi and the provision for reading conditions directly from the microscope. However, we also have a utility for suspending that communication for certain operations. In that situation, the last known conditions get stored with the spectra. If someone suspends communication while the beam is turned off, an accelerating voltage of zero is the last known value. That plays even more havoc with ZAF corrections than does a wrong take-off angle. EDS can produce some pretty decent results, but a good amount of care in setup and collection is necessary. Just because results are easily produced with the press of a button doesn't mean they are right. Warren Straszheim <wesaia@iastate.edu> 15 Feb 2007

SEM - shorted lead wires - current contrast?

We have a shorted biomedical lead wire. We've used test equipment to verify the short. What we would like to do is to visually identify the location of the short - in the SEM. The lead wire consists of 4 ea ~1 mm insulated wires that are then sheathed in insulation. We can sacrifice the outer insulation but need to retain insulation on the inner wires. I've imagined that if we power the cables inside the SEM, we might see current contrast that would divert from one conductor to another at the short. Is this experiment as easy as; building a vacuum feedthrough, connecting a external bench power supply wiring the cable to the feedthrough inside the SEM turn it all on and... Wah lah! Current contrast? Probably not that easy, so what am I missing? Owen Mills <opmills@mtu.edu> 16 Mar 2007

Thinking about your problem, I don't think that SEM is the answer. When you run current through the wires, it will generate heat, not electrons. If your SEM has CL, then it probably would work. I think the only practical (but potentially destructive) method is to pass enough current to cause the shorted area to heat up and burn. Or, run as much current as you can such that the wires do not burn and use an IR laser temperature gun and scan the wires for a drop in temperature. Just at this point is past the short. The amount of current needed is of course dependent on the resistance of the input end of the wires. Gary Gaugler <gary@gaugler.com> 16 Mar 2007

Real-time x-ray imaging will give you the answer you need. Many universities and commercial failure analysis labs have the capability. John Chandler <jpchandl@mines.edu> 16 Mar 200

SEM - lines on slow scan/capture

We have an Hitachi S3000N SEM. We have intermittent lines appearing across our images, mainly sourcing in bands from brighter areas, all the way across the image. This has got much worse over the last few weeks. We have ruled out electrical interference & vibrations. Has anyone got any ideas? Tanya Hayes <tanya.hayes@northampton.ac.uk> 23 Mar 2007

If you have ruled out simple charging effects then maybe you have a similar fault to us. We have a similar intermittent problem on our S3000N which is associated with fluctuation in the filament emission readout. It doesn't seem to be specific to either high pressure or high vacuum mode but sometimes will stabilize if run at a higher voltage and dropped back down - makes it seem like minor contamination. Generally this "flickering" effect gets progressively worse with the age of the filament (but is it an effect of age or is the flickering affecting the life of the filament?). The trouble is the effect could be self-fulfilling because the shortened filament life increases gun contamination and so may help to induce the effect. The erratic nature of the problem has made it difficult to solve or indeed know if it has really gone away. I had hoped that it might either go away or deteriorate to a point where the cause could be found. Sorry - no real answers and I can't be sure from your description if we even have a similar fault. But I will be interested in any progress you make or suggestions from the readership. Oh one thing that seems to make the problem worse (although may not be the only cause) is that the top of the Wehnelt cap can slowly work a little loose and although it may not be the only cause just gently nipping it tight can help. Good luck and if there's anything else that you need help with I'm sure I can be equally as informative (or not). Malcolm Haswell <malcolm.haswell@sunderland.ac.uk> 23 Mar 2007

Since you say it starts over bright objects, and is worse at slower scan rates it sounds like charging to me. A couple of suggestions: Have you changed to a different type of specimen lately? Go back to an object you have successfully imaged before, or one that should have no chance of charging issues - like a calibration grating, or stick a copper TEM grid to a stub with silver or graphite paint, even just the aluminum stub surface (rough up with sandpaper to give a coarse texture), and see if it is still present. If gone, it was charging and it could be something in the specimen type (round objects or powders have contact/grounding issues and will be worse) or something in the prep - maybe something in the sputtering? Does the sample look reasonable - like it actually got

sputtered? Note that samples will look different for the same sputtered layer if smooth vs. textured, white, etc. If it is still present with samples that absolutely should not charge, then it is in the instrument; is the stage grounding good? I had bands all the way across, not just from prominent bright details, and it was the spring contacts holding the filament - weak/oxidized - causing fluctuations. If you can rule out the sample, it could be any number of instrument related problems. But the "sourcing in bands from brighter areas" - if I understand your meaning -sounds like charging. Dale Callaham <dac@research.umass.edu> 23 Mar 2007

I had exactly the same problem on my S3000N last year. Because it persisted through filament and gun changes I knew it wasn't Wehnelt instability. I checked the stage grounding, the BNC cap for the stage ground and used a pure metal, conductive sample and still the problem was there. I checked the objective and final aperture for dirt. I cleaned the column and nothing seemed to help. I noticed the problem did not show up in the BSE imaging, so then I looked at the secondary electron detector itself. When I removed it, the cap over the fluorescent button was a bit loose and the button itself was charging up. I tightened the cap and put a small dab of conductive paint in the corner to make sure there was a path to ground for the electrons that hit the button. I have not seen the problem since. The front of the button has aluminum on it for grounding, but if there is a crack or not good contact between the button and the rest of the SE detector, charge can build up in the button itself, because it is made of a non-conductive plastic. I hope this works for you. Mary Mager <mager@interchange.ubc.ca> 23 Mar 2007

Well done, Mary. The give away here is the discharge line will have a little dart on it when it is scintillator discharge. Sure you get white lines just like charging but in this case the little dart is displayed on the lines. Steve Chapman <protrain@emcourses.com> 23 Mar 2007

I like Mary's solution. I just wanted to mention a few things about the "button". I assume that this Button is in fact the Scintillator that converts the incoming accelerated SE into light that can then be conveyed to and amplified by the PMT. At one time the scintillators were made out of fluorescent plastic but they had quite a short service lifetime (100 hrs) and only about 10 hours if used with high beam currents (nano-amps). The problem was the immensely high radiation damage as the incoming SE signal, now accelerated to about 10-15kV, smashed into the outer few microns of the plastic. Later, manufacturers switched to powdered-phosphor-deposited-on-glass scintillators (usually P-47). This inorganic scintillator gave a much longer service lifetime (1,000s of hours). However, as the powder was an insulator, the deposited powder layer had to be covered with a floated-on carbon or Formvar film and this was then coated with Al to provide conductivity. Don't put the Al directly onto the powder; it keeps the light from getting out of the grains. Sometimes the glass-blank below was given a transparent "NESA" coating. So now the service lifetime limitation became the Al-coated films. They can be damaged by the incoming ionizing radiation and also by mechanical forces associated with vacuum cycling, especially if any air gets behind the film. The point of this whole rant is that, if the film over the phosphor is "cracked" or otherwise damaged, you will lose a lot of signal. SE may land, but if the surface is negatively charged by previous SE, they will not land with enough energy to make much light and hence the SE signal will seem weak. This can happen so slowly that you don't notice it unless you occasionally calibrate your SE signal from a known clean conducting specimen (Si wafer?), with fixed kV, working distance, etc. A drop of silver may help but if the paint covers the center of the scintillator where most of the signal arrives, it will prevent the SE from reaching the phosphor and making light. *i.e.*, use the paint sparingly and only around the edge. James B. Pawley <jbpawley@wisc.edu> 23 Mar 2007

Electron beam simulation

I am trying to simulate electron beam heating in the SEM. I am sure this is not a new topic and perhaps lots of people had done some work on it. I am totally new to this area so like to check if anyone has good journals to recommend? In my simulation, I input a figure for the probe current density (taken from some journals), I inevitably get melting. I am still trying to verify this. Can anyone point out to me a typical figure for probe size, current and perhaps even current distribution equation for a TEM probe? Tan Thiam Teck <tttan@simtech.a-star.edu.sg> 04 Apr 2007

The current density may be high but the current itself is very low because you have essentially a point source in the specimen. You are right; it has been done many times. Except for really good thermal insulators (Styrofoam?) the heating is negligible (|--1°C) for beam currents of 10 to the minus 10 amps or lower. See Scanning Electron Microscopy by Oliver Wells (1975). Damage is usually due not to heating but to "radiation damage" cause by the fact that most of the energy is deposited in "lumps" of more than 20 eV each (*i.e.*, large enough for one "lump" to break a covalent bond). This is large and complex topic and is the reason that it is very hard to make images showing better resolution than 3 nm of any covalently-bonded material (*i.e.*, all biology). See: Electron Crystallography on the web and authors such as Robert Glaeser, Wah Chiu, and Ken Downing. Jim Pawley<jbpawley@wisc.edu> 07 Apr 2007

Microscopy AND Microanalysis

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Volume 13, Number 3, June 2007

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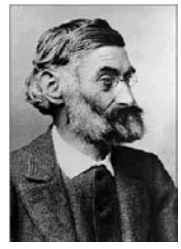
HUMOR



Dear Abbé

Dear Abbé,

My colleague and I have been having an argument about Antonie van Leeuwenhoek. We are hoping you could resolve this problem. Did Leeuwenhoek have a glass eye?

Mystified in Michigan

Dear Mystified,

Gott in Himmel, if I had a Deutschmark for every time I have been asked this question I could annex the Sudentanland. The truth is that Antonie von Leeuwenhoek did NOT have a glass eye. It was made of polished quartz. van Leeuwenhoek's microscope used a single lens design that had such a short focal length he had to press the lens against his cornea in order to focus it. Due to a childhood accident in which von Leeuwenhoek shot his eye out with a Red Ryder cross-bow (this was before BB guns) he had a quartz eye that fooled all the ladies into thinking that he was actually making eye contact. Because of this he was able to see things that would not be seen again for two centuries. This included bacteria, protozoa, and Paul Lynde actually giving a straight answer on Hollywood Squares. Today, one can achieve the same resolution. Simply replace your Visine with a bottle of immersion oil (R.I. = 1.30) and you too will be able to see the things that made von Leeuwenhoek dance the Hokey Pokey.

Dear Abbé

My coworker, "Bob", keeps trying to help me see the stereo pairs he produces. Unfortunately I've never been able to see things in three dimensions. Bob insists if I try hard enough, I should be able to do so. Any tips, hints, or helpful suggestions would be most appreciated.

Two Dimensional in Dubuque

Dear Flat Lander,

Ach! "Stereo pairs" always bring to mind that year as a University student when I dated the Blücher twins. But I digress. As you have been impressed upon by your persistent friend, 3D viewing can be very important when evaluating the spatial relationship of structural data. A bigger problem is the name calling you will endure from your apparent handicap. Even so, one shouldn't try too hard to see the three-dimensional view provided by the stereo pairs. Your eyes might get stuck in the cross-eyed mode which will decrease your ability to find your mouth while drinking. Which reminds me of the time I began seeing in 4D after a particularly good evening of too many Danziger Doppelbiers with my friend, Hans Delbrück.

Nothing is too perplexing or numinous for Herr Professor! If you need assistance with nagging doubts or persistent petulance, please contact his administrative assistant at jshields@cb.uga.edu.

MSA Local Affiliated Societies News and Views

Over the last two months many LAS have held their spring meetings with many requesting a Tour Speaker or financial support through the Grants-in-Aid or Special Meeting Support programs. Before you know it, summer will be upon us, which means we will be heading to Ft. Lauderdale in early August for Microscopy and Microanalysis 07. Along with the numerous organizers needed to consistently ensure a successful M&M meeting, it takes many local microscopists to volunteer their time on the Local Arrangements Committee (Ron & Dale Anderson, Co-Chairs) to guarantee a memorable meeting. This year the Florida Society of Microscopy (FSM) has stepped up to the plate while also finding the time to hold a spring meeting and provide this column with the first LAS historical profile. I want to thank Betty Loraamm, FSM Secretary/Treasurer, for compiling and sending this article along with Anthony Greco and Luisa Amelia Dempere for their contributions.

Lou Ross, MSA-LAS Director; rosslm@missouri.edu

Florida Society of Microscopy

"In April, 1980, an Electron Microscope Club was established at the University of South Florida (USF) with the purpose to promote, foster and maintain high standards of electron microscopy in research, clinical, and industrial areas and to enhance technical skills and ideas. The Departments of Anatomy and Pathology in the University Of South Florida College Of Medicine sponsored the new club and provided funding to support a series of speakers and a workshop on current techniques for high resolution Electron Microscopy (EM). Dr. Fritiof S. Sjostrand conducted the first workshop." From the FSEM Sixth Annual Meeting Proceedings.

Archived 1984 historical pictures (below) of a social gathering of FSM members at the USF Hillsborough River Park in Tampa, FL. Johannes A.G. Rhodin (author of the "An Atlas of Ultrastructure", 1963, and FSEM founding father) can be seen wearing dark sunglasses in the third photo.

In 1981 the club elected to form a statewide organization named "Florida Society for Electron Microscopy" (FSEM) and attracted members from both life and material sciences. It held its 1st annual meeting in March of 1983 at the Ramada Inn, Tampa, FL inviting ultrastructural research talks by students, technicians, faculty, physicians and industry representatives. Over 100 people were in attendance along with 20 representatives of microscopy and vacuum system equipment companies displaying their products.

Over the years FSM has presented workshops on immunolabelling methods for electron microscopy, cryotechniques, digital imaging in electron microscopy, morphometry and stereology, scanning tunneling microscopy, energy dispersive x-ray microanalysis, and confocal microscopy.

In 1991 we held a joint meeting with the Louisiana Society for Electron Microscopy and Southeastern Electron Microscopy (SEEMS). Though grants-in-aid from EMSA (now MSA), FSM was able to fund meeting expenses for several student and technician members. In 1993 FSEM changed its name to FSM following the footsteps of MSA to reflect its members' interest in all the microscopies.

In 1996 FSM hit its low point with its members and officers losing their stamina to conduct the annual meeting. Lucille Giannuzzi, FSM's Material Science Director-at-Large Officer, suggested that FSM hold a joint meeting with the Florida Chapter of the American Vacuum Society (FLAVS). FSM was reenergized! We have now celebrated our 10th joint annual meeting with FLAVS at the University of Central Florida's Student Union in Orlando, FL.

The Florida Society for Microscopy (FSM) held its annual joint symposium in conjunction with the Florida Chapter of the American Vacuum Society at the University of Central Florida in Orlando from March 11-16, 2007. Invited talks included a seminar by Dr. William Landis of Northeastern Ohio University (an MSA Tour speaker) on the "Effects of Gravity on Cultured Bone Cells" and a marine technology talk on a newly designed "Autonomous Microbial Genosensor" by David Fries of the University of South Florida. Maria Palazuelos of the University of Florida completed the microscopy session with a seminar on the "Characterization of Nanomaterials for Nanotoxicity Assessment: Resolving Nanoparticles in Complex Environments". The MSA-LAS Tour Speaker program has given us a way to bring recognized leaders in microscopy to speak at our annual meetings.

The symposium was attended by more than 190 participants including faculty, students and industry personnel. For the technical program we welcomed speakers from 9 Universities, 5 National Labs and 4 Companies. Twenty two vendors from all over US displayed their latest products, and sixty four students presented posters and entered the Student Poster Competition.

We are especially indebted to our loyal industrial corporations for their support over these past 27 years. Our hope is for FSM to continue to offer its members and the community of microscopists a place to meet and exchange ideas and skills for the betterment of the microscopy world. FSM members have joined with the Local Arrangements Committee for MSA 2007 in Fort Lauderdale this summer and are busy attending to the many details involved in hosting the annual meeting this year. ■