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

Selected postings from the MSA Microscopy Listserver (listserver @msa.microscopy.com) from 10/10/03 to 12/10/03. Postings may have been edited to conserve space or for clarity.

Image Processing – Scanning and Newton rings

Does anybody have experience on how to get rid of Newton's rings on scanned TEM images? I use a Minolta Dimage Scan Multi Pro and I'm quite pleased with the quality of the scanned images except Newton's rings sometime appear on them. Sergey Prikhodko <sergey@seas.ucla.edu>

A few decades ago I made many 3.25 x 4 inch lantern slides (pre-35mm slide presentations). To eliminate Newton Ring formation between the glass slide and the glass cover, we put a frame of paper between them. I do not know the depth of the focus plane in your scanner, but if it is not necessary to have the negative in direct contact with the scanner plate, you can easily cut a rectangle out of a piece of paper or thin cardboard, slightly smaller than your negative, put the negative on top and try it out. Perhaps a top frame is also needed. If this is true, a file folder can be used. The scanner that I currently use can accommodate a plastic negative holder that was made for it. It must be at least 1 mm from the glass plate to the negative surface and the image is still in focus. Pat Stranen Connelly <psconnel@sas.upenn.edu>

LM – Mercury bulbs

A user of the lab has asked me to separate urban myth from reality regarding HBO bulbs used for fluorescence microscopy. What is the difference between a 50W bulb and a 100W bulb? He says he set up a demo, identical except one scope had a 50W bulb, the other a 100W bulb and he didn't think he could tell much difference. Do bulbs have a finite life, i.e., should they be changed at some maximum hours even if still lighting up and appearing OK? Is it true that bulbs with many hours may explode? Jon Krupp <jmkrupp@cats.ucsc.edu>

The 100W HBO lamps put out more light. There are issues of lamp alignment and condensing lens choices for the lamphouse which can have a dramatic influence on the amount of light that reaches the specimen. If your fluorophore is bright, you may be able to use the 50W lamp and not notice much difference between the two. If there is not much fluorescence, you may not be able to see it at all with a 50W lamp. The 100W lamp is much more stable then the 50W lamp. There is nothing more irritating then using a microscope with the light intensity flickering wildly. The 100W lamp is not likely to do this. The rated life of the lamps is 100 hours for the HBO 50W AC and 200 hours for the HBO 100W. Osram says that you can safely exceed the rated life by 20% without danger of bulb explosion. The bulb will explode if run too long, based on several factors. The rated life is determined by the factory to be the point at which the light output has been reduced by 30% from the initial output of a new lamp. A lamp driven by a properly operating power supply should look new when you change it based on maximum allowable hours. How dark it is should not be a criterion. Some people run their lamps much longer then it is safe. They may have power supplies which help them do this or they may get lucky. If not, the damage done inside the lamphouse by an exploding lamp will cost $300-500 to repair. You can purchase a lot of lamps for that much money and also avoid the dubious pleasure of breathing poisonous mercury fumes. These lamps have significant amounts of mercury in them, please dispose of them properly. The lamps cost the same amount, but the life is different. You should be paying under $100 US for them. Shop around. It seems like quite a bargain to get twice the power of the 100W, for half the cost of a 50W (based on lamp life). We have had the best luck with Osram lightbulbs based on the 3,000 or so we have purchased over the last 20 years, and have found no difference in price which would warrant using another brand. In general we have found no significant differences in performance either. The lamp must be run for 20 minutes at a minimum every time it is turned on or it may be ruined. This is the only use issue with this type of low wattage mercury lamp. The newer power supplies are different then the old ones some people remember and so stories of what "used" to work are of little help today. The newer power supplies will run every lamp to its rated life if the power supply is working correctly. We see lots of power supply problems which cause short lamp life. HBO power supplies seem to be problematic for some reason. If you purchase a new system and experience short lamp life I would have the supplier of your system install and align a new lamp for you (showing you the proper way to install it). If the lamp still does not reach its rated life, insist that they swap out a known, good power supply for you to try. In my experience that invariably fixes short lamp life problems. David Burton <dburton@nwlink.com>

EM – phase separators for liquid nitrogen

A recent posting requested asking how phase separators for liquid nitrogen tanks work led to these interesting replies:

There are also good economic reasons for having a phase separator: you will get far more of the liquid into your container with one than without. It pretty much eliminates the atomization that occurs without one. In my experience, a sintered bronze separator should be available and work well for years. What is coming down the delivery tube is a boiling mix of gas and liquid at 40 to 200 psi. If it exits from the open tube, there is a large amount of atomization and subsequent evaporation. In other words, most of what comes down the tube never makes it into the container. The phase separator is able to cause a lot of the fine mist to coalesce and gravity pulls the liquid to the bottom end. The gas is free to escape from the upper part, less its liquid load. The yield for the liquid part is much greater with it just running freely into the container (without pressure). Your example shows how important it can be. I'm assuming that the valve was opened the same amount and the feed rate from the bulk tank was the same. That means that you used less than half as much from the bulk tank using the separator. And it saved you time in the bargain. Ken Converse <qualityimages@netrax.net>

If one is using an LN2 tank at 40 to 200 psi that is a major part of the liquid delivery problem. A tank intended to deliver liquid, as opposed to gas-phase coolant, should be a low pressure tank that maintains the liquid only under about 20 psi, enough to boost it out the nozzle and little more. Most delivery services can provide either low pressure (~20 psi) tanks or high pressure tanks. The typical 110 L size of each is very similar in appearance except for the plumbing and pressure regulation apparatus at the top. The high pressure tanks are preferred by users of temperature cycling equipment where

the objective is to maximize cooling throughout a protracted discharge from the tank without delivering liquid into the test chamber. It can be virtually impossible to collect the liquid effectively from a high pressure tank. John Twilley <jtwilley@sprynet.com>

TEM – staining grids

Does anyone have a method to jazz up the uranyl acetate (UA) followed by lead citrate post-staining procedure? I am working with some tissue that just doesn't seem to want to stain. It looks very dull in the scope. I'm using a saturated aqueous UA then Reynold's lead citrate. Should I try UA in 50% ethanol rather than aqueous UA? Beth Richardson <beth@plantbio.uga.edu>

You will find very good results with 2% UA in 100% methanol followed by the usual lead citrate protocol. We stain with UA for 8 minutes and then wash in 70%, 50%, and 30% methanol followed by double distilled water and then stain with lead citrate for 10 minutes. The type and hardness of the resin used for embedding can cause some variation. We get very good results in plant tissues with the above technique. Francisco Freire <sme@sgi.ulpgc.es>

I use 70% ethanol with UA and it appears to yield better contrast than the aqueous solution. Mary Engle <mgengle@uky.edu>

You didn't mention the tissue type or the resin, but if its Spurr's epoxy, that can make staining of embedded tissues more difficult, especially with Reynold's lead, I think. Or maybe the tissue type just doesn't pick up much stain. I don't usually use Reynold's stain here, but prefer to use the Sato triple lead stain (lead citrate, lead nitrate, lead acetate), which I've been using for many years. The way I make it up, its very stable and gives excellent staining, usually with no lead precipitate contamination, though if its older than 2 months, I may use a 0.2 micron filter as a precautionary measure. A 100 ml batch typically lasts 3-4 months. Before the Sato lead, I stain with 3% aqueous UA for about 20 minutes, room temp. But you may need to cut the UA with methanol and/or elevate staining temperature as others have suggested on this thread for your special case. Gib Ahlstrand <ahlst007@tc.umn.edu>

I have used ethanolic UA for almost 35 years, with very good results. The procedure calls for saturated UA in 50% ethanol, stir for 5 minutes, stain for 10 minutes in reduced light. Depending on how much stain you need at a time, you can make it up in as little as 5 ml preparations. It takes just under 200 mg in 5 mls to get a saturated preparation. I make it up fresh and filter it through a 0.2 micron micropore filter just before use. It keeps OK in glass for several days. I've never tried to keep it longer. It keeps very poorly in disposable syringes, only 2-4 hrs, the rubber on the end of the plunger reacts with the solution. The reference for the procedure is: Stempak JG, Ward RT (1964) An improved staining method for electron microscopy. J. Cell Biol. 22:697-701. Paul R. Hazelton <paul_hazelton@umanitoba.ca>

If you are using an Epon type embedding, saturated UA in 50% ethanol would be a great help. Some cautions in working with it (besides the radioactive chemical safety ones) are: 1. to really keep

it in the dark, even while in the staining dish. 2. Make it in small batches (5 ml in a small vial works well) because it looses its "umph" after just a few days. 3. No need to filter the stain if you let it settle for about 15 minutes after shaking and the stain is taken gently from near the top of the vial so as not to disturb the bottom crystals. A second place for lack of contrast in staining is to leave the lead stain on too long. The metal will be leached back into the solution. The samples I work up are usually block stained in 0.5-1.0% aqueous UA in the refrigerator overnight after fixation. My section staining procedure is to place a dry grid onto a drop of UA stain in a Petri dish on Parafilm for 8 to 10 min. The lid of the dish is covered with cardboard and opaque tape. Water rinse several times and put the grid into a large drop of water also on Parafilm in another dish and leave the grid there until all grids are washed. Transfer right out of the water drop into a drop of lead stain in a third covered dish for about 1-2 min. then wash well before drying it down. Although I have used Reynold's lead stain in the past, the lead stain that I am using now is lead citrate as described by Aly Famy in Proceedings of the 25th Annual EMSA Meeting (1967)- 50 ml cooled, boiled, distilled water + 1 dry pellet of sodium hydroxide, after it is dissolved add 0.2 g (reference states 0.25g) lead citrate. I use a polypropylene plastic 50 ml centrifuge tube so it can be discarded later and store the staining solution in several syringes stuck into a large stopper to keep out the air and keep it in a cabinet to limit light exposure. Pat Connelly <psconnel@sas.upenn.edu>

It sounds like this is a sample that would benefit from high pressure or plunge freezing followed by freeze substitution. That way you would minimize collapse and pH would not be a critical factor. Even adding fixative to the freeze substitution solution wouldn't matter since you would not be buffering it. Debby Sherman <dsherman@purdue.edu>

TEM - negative staining

I have done negative staining (1% PTA) on a budded virus for TEM and some of the virus species appears white while others, on the same grid, have a darker appearance. Does anyone have any experience of this phenomenon? Is this depending on if the envelope membrane is lost or not? Pernilla Nevsten <pernilla.nevsten@materialkemi.lth.se>

This was pretty common in our studies of the Mammary Tumor Virus. Are you seeing osmotic artifacts as well? In our studies of MTV, the virus often appeared as a "head" with a membranous tail. You can check some of the other ways we prepared the virus in: Cancer Research (1975) 35:740-749. Joel Sheffield <jbs@temple.edu>

What you are probably seeing is both positive (dark virus) and negative (light virus) staining. True negative staining gives a light particle surrounded by stain which, due to the scattering of electrons, appears darker. You will often also see some darker areas of the particle that have trapped some stain. Positive staining is when the particle itself has absorbed the stain and appears dark while the background is light. Usually there is very little detail in positively stained particles. Positive staining often occurs when the amount of sample material is very low. In this case, the hydrophobic nature of the film surface results in the stain just rolling off. You can see this happening if you put a droplet of stain on the grid and, when you go to wick it off with filter paper, it all comes off leaving an apparently dry grid behind. You can reduce this problem by glow discharging the grids in a vacuum evaporator just prior to making your samples. This acts to change the charge on the grid surface and make it hydrophilic. This effect does degrade quickly so grids should be used within a few hours. You can glow discharge grids more than once. The alternative is to use a sample with sufficient concentration of particles so that there is enough material to hold the stain. It is not unusual to find areas of both positive and negative staining on the same grid based on where the sample has accumulated. The nice thing about negative staining is that you have a fairly homogeneous sample so usually you can hunt around for just the right sample and stain distribution. Debby Sherman <dsherman@purdue.edu>

Virus will appear differently (some darkly stained, some lightly stained) for various reasons: 1. Stain penetration: sometimes the integrity of the virion is disrupted (mechanically, chemically) so that the negative stain can penetrate the particle and deposit inside the virion giving it a dark appearance. 2. Defective particles: in most replicating virus there will be a certain percentage of defective (empty) particles. These are generally "leaky" and permit stain to penetrate. If the population contains too many defective (non-infectious) particles, this leads to a phenomenon called defective interference and could eventually lead to the loss of viability of the virion (infectious particles). 3. Variability in staining: negative stains are rarely exclusively negative and there will always be some degree of positive staining taking place with the stain. Uranyl acetate, for example, often reacts intensely with DNA if it is accessible and PTA sometimes stains polysaccharides (external components of membranes). John J. Bozzola <bozzola@siu.edu>

TEM – negative staining

I have a project from a faculty member who is studying a protein, the monomers of which form a ring. I'm using 1% uranyl acetate as the negative stain. This was the method used in the background information they brought to me. Is there an even finer grained negative stain? I'm looking for a way to improve the visual quality of the material. The operating conditions of my TEM (a Philips 410LS with tungsten filament) are 80Kv and 100Kv (the limit of my TEM); aperture sizes are condenser 200 micron, objective 20 micron, Spot size 2, zero degree tilt. I'm trying to photograph in the range of x240,000 to x500,000 (the upper limit of my TEM). I would appreciate any suggestions to improve the image of the samples either by way of specimen preparation or operating condition of the instrument. Would metal shadowing be worth trying? Tom Bargar <tbargar@unmc.edu>

Nanoprobes offers a vanadate-based negative stain, NanoVan, which several users find gives a very fine grain structure - check the paper by Gregori et al (1997) J. Biol. Chem. 272:58-62 for an example. Reprint: http://www.jbc.org/cgi/reprint/272/1/58.pdf Reprint of our 1994 MSA abstract: http://www.nanoprobes.com/MSANV.html Since it is based on a lower Z element, the staining is lighter. We also offer a tungstate-based analog, Nano-W, for denser staining. Rick Powell <rpowell@nanoprobes.com>

Since Rick Powell covered the stain half of your question, I thought I'd take a stab at the other half. The negative stain consists of clumps of material typically ~1 - 2 nm in diameter, so the magnification you're using results in blobs of stain ~1/4 -1 mm on

your detector. If you lower the magnification, you will have smaller, but still visible clumps of stain, and spreading the beam can reduce effects from heating and radiation damage; additionally, you will get a larger field of view. Even with a W filament, you are concentrating a large number of electrons on a very small sample area and few electrons outside this area, so you can cause motion even of very heavy atoms. Bill Tivol <tivol@caltech.edu>

I have a Philips CM-12 TEM. Your operating conditions seem OK to me for the magnification range you indicate, except for the spot size. Drop it down to spot size 4-6 and see what you get for particle resolution. Take a photo at your spot 2 setting, and then shoot same particle at lower spot sizes for comparison. I have photographed carbon nanoparticles at those magnifications and the smaller spot size definitely improved the resolution of the details. Your exposure times will increase so you must have a stable sample. You can also kick up the emission level of the electron gun, or even set tungsten filament tip a little closer to Wehnelt cap (but not so close that you get arcing) to increase the brightness at those smaller spot sizes. Use your cold trap. After a half-hour cool down, you will reduce any beam induced contamination of the particles you are looking at. Gib Ahlstrand <ahlst007@tc.umn.edu>

Another negative stain to consider is uranyl formate. It is a bit fussy about staying in solution, so you need to make it up just prior to use. Don Gantz <gantz@bu.edu>

TEM – Immunolabeling

I have an investigator who is carrying out EM immunolabeling on isolated dispersed filaments. The labeling is carried out on the grid but the filaments are not visible without negative staining. It would appear that the labeling procedures deposits considerable material on the grid which shows up as a thick layer and this obscures the filaments. Is this to be expected? Is it an inadequate washing procedure after labeling? Christopher Gilpin <christopher.gilpin@utsouthwestern.edu>

The solutions wouldn't ordinarily be dirty enough to deposit anything. I think he/she may be using too concentrated an antibody solution. Also, leaving the antibodies on for too long a time might accomplish the same thing. Carol Heckman <heckman@bgnet.bgsu.edu>

I use to do immunolabeling followed by negative staining on virus particles and did not have this kind of problem. You can see the procedure I followed in this paper : Spehner, D., R. Drillien, F. Proamer, C. Houssais-Pecheur, M. A. Zanta, M. Geist, K.Dott, and J. M. Balloul (2000) Enveloped virus is the major virus form produced during productive infection with the modified vaccinia virus Ankara strain. Virology 273:9-15. Spehner Daniele <daniele.spehner@efs-alsace.fr>

SEM – Au vs Au/Pd coating

The following replies were made in response to a question asking if using a less expensive Au target, instead of a Au/Pd target, in a sputter coater would cause a significant difference in the image quality obtained with a high resolution SEM or FESEM. In addition, the individual wanted to know if one could recover any lost image quality by using Au/Pd to re-coat a sample previously coated with Au.

It is my understanding that Au/Pd does a better job at coating rougher surfaces because of its smaller grain size. It may depend on what you coat. Ron L'Herault <lherault@bu.edu>

My understanding is that gold coating produces a coarser surface coating because Au alone nucleates on the surface as it is coated and islands of Au limit the image quality in high resolution SEM. The Au/Pd alloy forms smaller, more numerous islands and so provides some improvement in resolution over gold. Simply coating a previously gold coated sample with Au/Pd will not improve the situation, but should make it worse because the sample will be coated with a thicker layer. I am surprised that you find much difference in price between Au and Au/Pd targets. In the UK, they are either the same price or I am sure that I have seen Au/Pd a bit cheaper. I think the problem is defining what you mean by high resolution in SEM, but normally this should be achieved by a good traditional SEM or certainly will be by a field emission SEM. I'm sorry I have no figures - the information I have just says Au/Pd is noticeably better than Au at high resolution. Instinctively I would guess the difference would be noticeable somewhere between 20k and 40k, but I would be interested to know if anyone could quote any real hard figures. There are now lots of alternatives to Au and Au/Pd which give even better resolution and I have seen some remarkable images taken with very low voltages on a high pressure field emission SEM - but I can only dream. Malcolm Haswell <malcolm.haswell@sunderland.ac.uk>

Depending on which XL-30 you have, there may or may not be a difference. If you are using the thermal SFEG, it will definitely make a difference. With SFEG or non-SFEG systems, if resolution is good enough at high magnification (>x75K), you can see the Au coating as a web-like structure on the surface. If you coat over this with Au/Pd or Pt, it will just make the structure more obvious. Cold/magnetron coaters with Au/Pd targets do a very good job laying down an ultra fine film. Pt works well too. I use a Denton Desk II coater for semiconductor work up to x350K. Au alone is not satisfactory even at x50K since the Au structure distorts the image. I have used an Anatech Hummer VII coater before and also got good results. So it would seem to me that a good coater with an Au/Pd target will do a superior job over Au for high resolution, high magnification imaging. Pt targets are more costly but are even better. Use a low current, high vacuum setting. I'm running the Denton at 20mA, 60mT for between 30-40 seconds. There are basic price point differences between Au/Pd and Pt. Pt is higher cost. However, keep in mind that many places sell targets with different thicknesses. A cheaper thin target will not last as long as a modestly more expensive thicker target. Used on a regular basis, my targets last over two years before needing replacement. The coating is only about 50-70A thick. My work ranges from x100 to x350K. Au/Pd works for all but the most demanding specimen is. If I were to choose one target, it would be Pt. I would use it for every specimen. But some specimens are better analyzed with EDS using Au/Pd, others with Pt. So it depends. The other factor is to keep coated specimens under vacuum storage when not being used. Invariably, there will be carbon polymerization that shows up as scan areas. These can be reduced by keeping oil out of the chamber and atmosphere away from the specimen. A small trap in the coater vacuum line helps keep the coater from backstreaming. Probably the ultimate coater is a turbo pumped system. But I have yet to find one that is user friendly enough and cost effective to justify purchasing. Gary Gaugler <gary@gaugler.com>