NETNOTES

Thomas E. Phillips, Ph.D.
University of Missouri
phillipst@missouri.edu

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TEM - Immunocytochemistry and Gold Vs Nickel Grids:

What is the advantage to using gold grids instead of nickel for post-embedding immunochemistry? Neil Medvitz <medvitz@pitt.edu>

There are several advantages which could be of greater or lesser importance depending on individual situations: a) When a pair of normal, antimagnetic stainless steel tweezers are used to hold a typical nickel grid during a typical immunoreaction, you have two dissimilar metals in contact in a low pH environment and you get a current flow (this is the basis of electrochemistry). And this current flow is thought to stunt the strength of the immunoreaction. I don't know to what degree that is published but this is what customers have told me over the years as to why they have concerns about nickel grids. b) Gold grids don't suffer from this problem and they are far more inert under these same conditions than nickel. But gold is softer, and the grids tend to be a bit less self-supporting and therefore more difficult to work with, whereas nickel is stiffer, and does not bend as readily, that being the reason why some workers, at the end of the day, prefer nickel. One can get around the electrochemistry issues when using Ni grids, however, if instead of using the normal antimagnetic stainless steel tweezers, gold plated tweezers are used. The gold plating acts as a passivation layer on the antimagnetic stainless steel tweezers, killing off the chances for an electrochemical reaction, also leading sometimes to corrosion product in one's samples. Charles A. Garber <cgarber@2spi.com>

Gold grids are a good choice for on-grid immunodetection when using conventional immunogold reagents with a size suited for visualization without enhancement (e.g., 10 nm gold particles). Gold grids are virtually chemically inert and thus won't easily interfere with components in incubation solutions. There is one exception: silver enhancement. Since gold particles act as catalysts in the deposition of metallic silver, gold grids may become covered with metallic silver as well, locally exhausting the enhancement reagents and leading to non-reproducible results. Nickel, as far as we have tested, does not seem to act as a catalyst for silver enhancement and in a practical sense is also chemically inert towards incubation solutions. In our experience handling such grids with non-magnetic tweezers (or better, with platinum loops) has never been a reason for doubting the immunocytochemical results. The main downside to using nickel grids is that they influence the electron beam and cause astigmatism making more frequent adjustments necessary while observing specimens in the TEM. Electrochemical phenomena may already occur whenever a metal surface is brought into contact with a solution. It does not necessarily involve a second (different) metal. Whether this actually results in a chemical reac-

tion depends on the redox potential of the components involved and whether the overall conditions are favorable for a reaction to occur. Jan Leunissen <leunissen@aurion.nl>

We've never had any trouble using nickel grids for immunocytochemistry. Copper grids sometimes can react with reagents, particularly if you incubate overnight, leaving blobs of copper sulfate. As for gold grids, they can be used for growing cells on support films because the gold is not toxic to the cells. However, I think gold is overkill for immunocytochemistry. Gold grids are expensive, especially when you are testing multiple dilutions and controls. We don't use forceps at all with our nickel grids. We use loops made with either nickel or gold wire similar to the wire you would put into the vacuum evaporator. We glue the wire onto sticks made from the plastic disposable loops normally used for spreading bacteria onto plates after cutting the plastic loop cut off. The loops are made with 1-2 inches of wire, wrapped around a drill bit just larger than the grid and twisted tightly to make a loop at one end. The other end is wrapped around the end of a plastic handle and glued with epoxy. The advantages are that loops are reusable, and there's less likelihood of bending the grids. Just be sure to wash the grids between solutions sufficiently. We use 5-6 washes in drops of buffer. At the very end of the staining protocol, we pick up the grid with forceps and dry it. Sara Miller <saram@duke.edu>

TEM - Lowicryl K4M Sectioning

I recently embedded some freeze-substituted skin samples with Lowicryl K4M. The blocks were cured under UV light at -35C for 2 days and at room temperature for several days. The blocks look fine. However, when I section them, the block surface always gets wet and the sections swell badly on the water. It seems like sections dissolving into water. I was unable to pick up any intact sections. I have used Lowicryl HM 20 before and it worked very well for me but I can get not good sections of Lowicryl K4M. Shanling Shi <shanling.shi@unilever.com>

You have discovered the well known downside of K4M but it can be overcome. Half the battle is to get the water level in your knife boat just right. We also find the sectioning characteristics (and I think how easily water jumps to the surface) also improves if we store the blocks in a desiccator overnight before we cut them. Don't use too slow a cutting speed since that promotes wetting of the surface. If you do get the block face wet, you need to dry both the block face and back of the knife before re-starting or it will wet immediately upon restarting. We prefer LR Gold for this reason but Lowicryl K4M has some features that make it superior for some antigens. Tom Phillips <phillipst@missouri.edu>

Regarding the sectioning and wetting of the block face, here are a couple of tips: (1) You will probably have to section the K4M with a very low water level in the boat, much lower than you are used to, to avoid water "jumping" up onto the black face. In fact, you can get fairly good sections when the reflection at the knife edge is dark, rather than the normal reflection you see when the boat is properly filled for normal sectioning. (2) If you are using a diamond knife and the edge is hydrophobic, overfill the boat on the knife to form a positive meniscus and let the knife sit like this for 15-30 minutes, then try to reduce the level in the boat to give a very dark reflection along the knife edge (meaning the water level is lower than normal). In fact, the first 2 or 3 sections may get lost in the dark reflection so that you can barely see them, or not see them at all. (3) In the past, I have also had good luck by doing the following: After facing and trimming the block, approach with your diamond knife with a partially full boat and a "dry" knife edge. On the final approach, with the microtome running and advancing, stop the microtome after the very first bit of the block has been cut (usually a partial section is cut). Then gradually bring the water level in the boat up until the water makes contact with the dry section sitting on the knife edge. At this point the water will creep under the section and lift it off the knife. The entire knife edge will probably not be wet, but the area where you are cutting will have water under the section and the knife edge in this area will be wet. Start the microtome again, and gradually add water dropwise between cutting cycles to slowly raise the level of water in the boat. This will usually work with K4M, and you should be able to get reasonably good ribbons from the block. (4) Remember, always cut with the water level lower than normal. This will prevent the block face from getting wet. Bob Chiovetti <rchiovetti@aol.com>

TEM - Diamond Knife Angles

I am interested in the advantages and disadvantages of a 35 degree diamond knife compared to a 45 degree one. 35 degree knives are reported to cause less compression but is this found in the real world? Tom Phillips <phillipst@missouri.edu>

35 degree knives are great for cryo-sectioning. On the other hand, they are more fragile and need more attention. For plastic sectioning, I would suggest: if you have only one knife - it should be a 45 degree knife. Sergey Ryazantsev <sryazant@ucla.edu>

We use 35 degree knives all the time for softer cutting materials at ambient temperatures, primarily clean polymers and biologicals. I highly recommend them. Bradley J. Huggins <hugginbj@bp.com>

The 35 degree edge will cut much more easily and with less compression but the downside is that the knife wears out faster. Charles A Garber <cgarber@2spi.com>

I have used both and found no difference on well fixed and embedded samples. I also tried them with Epon, Spurr's, LR White and Unicryl resins and in each case the results were same. I still needed to stretch the sections with either a heat pen or solvent. Neelima Shah <shahn@mail.med.upenn.edu>

I use a 35 degree knife for Epon and cut at around 4.5 degrees on my ultramicrotome and get great sections with no compression. I also use the same knife to cut Lowicryl HM20 and get beautiful sections with no compression when I set the microtome at 2 degrees and increase the cutting speed a bit. I used 45 degree knives for 20 years and this new 35 degree knife for a year and a half. I think the 35 degree knives are better. William Oxberry <william.oxberry@downstate.edu>

SEM - Cleaning A Penning Gauge

I need some advice on cleaning a penning gauge. I have an Edwards Model CP 25-K, on a Cambridge 360 Stereoscan. There are some very stubborn deposits, especially on the three inserts. Robert Kayton <kayton@ohsu.edu>

Both the cathode cups (part #D145-33-007) and anode post (part #D145-33-006) can be replaced and are available from your local Edwards dealer. They can also be cleaned by abrasion with SiC paper and reused but beware that the anode has been surface treated and will not last very long after abrasion (about 6 months) so it is better to replace it. For the cathodes and the body of the gauge, I use SiC paper and wash with alcohol afterwards. I only

replace the cathodes when they are really shot. Note the orientation of the cathode cups when you take them out. Ron Doole <ron.doole@materials.oxford.ac.uk>

If you have access to a bead blaster (a sand blaster that uses fine glass beads), it's a lot less work than SiC. Any aluminum parts should be done with a reduced pressure of 35-40 psi or you may find that the aluminum disappears pretty quickly. If the stainless steel parts don't clean up at that pressure, you can go back up to 100 psi or so. Be sure to mask any sealing surfaces with masking tape before blasting. I don't believe there are any screw threads inside your gauge, but some have threaded parts. Male threads should be masked with masking tape and female threads should have a properly sized machine screw inserted to protect the threads. Ken Converse <qualityimages@netrax.net>

The instrument I used, called an "air eraser", offered a choice of glass beads, corundum, or, I think, starch grains (in any case, something pretty soft) to use as the abrading particles. The soft abraders could be useful for aluminum; however, the dirt may be tougher than the aluminum, making the process unsuitable. The air eraser is ~$100, and it's useful for a number of tasks. Bill Tivol <tivol@caltech.edu>

With the correct choice of abrasive size and hardness, and the right choice of operating pressure and distance from the nozzle, abrasive cleaners can be helpful tools. However, in using one you should be aware that this microscopic "shot peening" of the surface has other effects on metals, including work hardening and distortion. For example, in microelectronics, bright-plated gold is notoriously hard to form a reliable polymer bond to. To overcome this problem I once worked with a process that required micro-abrasive roughening of the plating surface in the bottom of a flat Kovar package about the size of a matchbook. In the course of 30 seconds of abrasive blasting, the thin bottom of this package would acquire a visible bow due to its lateral expansion within the confining sidewalls. It may seem counter-intuitive, but the direction of the bow was toward the abrasive jet rather than away from it, due to expansion of that surface relative to the unabraded rear. John Twilley <jtwilley@sprynet.com>

TEM – En Bloc *vs.* Staining Sections

The following postings were in response to a request asking for comments concerning the usefulness and advantage of doing en bloc staining of biological tissues as compared to heavy-metal staining of grids with thin sections.

Uranyl acetate stabilizes lipids, membranes, and nucleic acids when used as a fixative. Studies have shown less lipid extraction by the organic solvents after uranyl acetate fixation. It also contributes somewhat to contrast, though not quite as much as Osmium Uranyl acetate is generally used after osmication for additional stabilization and contrast. It can be used in veronal acetate buffer, aqueous solutions, or in alcoholic solutions. We routinely use uranyl acetate en bloc in pathology and virology samples except in cases where one needs to see glycogen deposits (e.g., glycogen storage diseases). Thus, we never use uranyl acetate in cases of heart, skeletal muscle,

and Ewing's sarcoma (if Ewing's is suspected). Uranyl acetate and lead, used as post stains, on sections add a general contrast to structures present. In this case, uranyl acetate does not contribute to fixation or stabilization because the structures are already embedded in resin. Sara Miller <saram@duke.edu>

I agree with Dr. Sara Miller's point that uranyl acetate en bloc staining helps to some extant in preservation of ultrastructure as well as enhancement of the contrast. However, it also has disadvantages. Uranyl acetate is toxic and sometimes causes background staining from crystal precipitation, especially when ethanolic uranyl acetate solutions are used. More importantly, it is time-consuming because it takes hours for an aqueous uranyl acetate solution to penetrate a sizable tissue block. Therefore, I would say it all depends on your tissue and purpose of your analysis. In my lab, we routinely do en bloc with samples of blood, tissue culture, virus, bacteria and other microorganisms, which are believed more vulnerable to solvents during dehydration, but not with solid tissue. We have examined a lot of samples from kidney biopsies and have been very happy with staining thin sections. We believe taking your time to have a sufficient osmication is much more helpful to your structure. Greg Ning <gxn7@psu.edu>

Image Processing - Removing Gradients

In doing SEM, I occasionally acquire images that contain a gradient. One example would be an x-ray map that I have acquired in an hours time. Typically the gun will drift slightly out of alignment and the end of the image is darker than the beginning. Another example is when I am collecting very low magnification images using the conventional SEM detector (Everhart and Thornley). The top left hand side of the image is much brighter than the bottom right hand side. I would like a routine that averages pixel values, say nearest ten neighbors, to create a new image based on just the averages. I could then perhaps divide the original image by the average image and in effect, normalize the original image. It would be great if I could do this within Adobe Photoshop. Since it is SEM at least we are dealing with just 256 gray levels. Ric Felten <smartech@optonline.net>

See Sedgewick's article in *Microscopy Today*, 11,1 Jan/Feb 2003. ...Editor

A simple recipe I use for leveling shading of SEM/TEM images and diffraction patterns in Photoshop follows: (1) Open image. (2) Duplicate layer (twice, to keep unaltered original). (3) Apply a Gaussian Blur filter to the top layer. Start with about a 25 pixel blur radius and adjust to get desired effect. (4) Invert the image contrast. (5) Select Overlay mode in Layers. (6) Merge down once. (7) Repeat this process 2 or 3 times as appropriate. You can record all the steps as an Action in Photoshop. This method works best with images having no abrupt brightness transitions, such as annotations. Larry Thomas <larry.thomas@pnl.gov>

The technique you are referring to is sometimes called "Unsharp Masking" and has been used by astronomers for a long time. It is used to get better contrast from differently illuminated areas, and it will do a background equalization as well. In general, you get the best results if you can take an image of the background by itself and correct the image with that background. In a TEM, that can be done by leaving any settings undisturbed and removing the specimen, then taking a picture of the background. I am not sure that can be done in an SEM. That leaves you with an artificial background correction. Unsharp Masking is one way to do this. Other possibilities include the calculation of a background image from areas where the background shows through. You also have to decide whether you want to divide or subtract. In most cases a division is the correct operation (for example, if irregularities in a TEM phosphor need to be taken out). Mike Bode <mb@soft-imaging.com>

Remove the long-wavelength intensity fluctuation from your images using a high pass Fourier Transform filter. This and other free background correction utilities are available in ImageJ at http://rsb.info.nih.gov/ij/. ImageJ is the most versatile 2D image processing and analysis software for those who do not require super-high speed. Paul Baggethun <paul.baggethun@alcoa.com>

TEM – Sulfur Embedding

Have any of you any experience embedding materials, especially rocks, in sulfur for subsequent thin sectioning? Rick Hugo <hugo@pdx.edu>

We have worked on embedding particles in sulfur for some time now. I have always thought to present this technique at MSA but just haven't gotten around to it yet. To the best of my knowledge, the idea to embed in sulfur was conceived by our colleague, John Bradley. Perhaps others have done so as well. We developed our own set of sulfur-embedding techniques and methods suitable to our particles. Embedding materials in sulfur is a wonderful technique to look at microtome sections since sulfur will sublimate in vacuum and you can be left with a section on a TEM grid that is free of embedding material. We study interplanetary dust particles (IDPs - typically 5 - 10 μm in size) which

are composed of 1000's of mineral grains, glass and carbonaceous phases, including organic materials. These particles are often highly porous and the normal infiltrating epoxy resins have always made it difficult for us to determine the carbon compounds vs. the epoxy, thus our need for this technique or other carbon-free embedding methods. Sulfur is a very difficult material to work with and requires fairly extensive experience and practice due to some unusual properties. For instance, liquid sulfur is one of the rare materials whose viscosity goes up as you heat it to higher temperatures (at least up to about 190 C); this is due to increasing polymerization of sulfur ions in the melt with increasing temperature. Our technique of sulfur embedding is to place a particle (say a 10 um interplanetary dust particle) in the middle of a clean glass slide. On a second clean glass slide we heat a tiny crystal of ultra-pure sulfur above its melting point of 119 C. The sulfur will melt and, after removal of the heat, will supercool forming a liquid drop on the glass slide. I like to have the drop diameter of 50 - 100 µm or less, otherwise the liquid sulfur may spontaneously crystallize. While observing under a good binocular microscope, the particle on the clean glass slide is lowered into the sulfur drop which will wet both glass slides. When the two glass slides are separated, the particle on the first glass slide will have a hemispherical liquid drop around it. At this point, you need to crystallize the supercooled sulfur which surrounds the particle by touching a small sulfur crystal to the drop which will create nucleation sites and initiate crystallization. There are other ways to do this besides what I have just described. Unfortunately, fractures may develop in the sulfur during crystallization which can lead to contamination and other problems later. Fracturing of the crystallized sulfur is a problem with this technique that we haven't found a satisfactory solution to yet. The particle in solid sulfur is then mounted with epoxy to a pre-cut epoxy cylinder which fits the chuck of the microtome. The sulfur mount must first be dislodged from the slide as the crystallized sulfur will want to stick to the glass. It can then be trimmed and microtomed in the usual way but you have to be careful as microtoming is more difficult than with epoxy resins due to the softness and fragile nature of crystallized sulfur but it can be done with practice. For us, this works best on small samples (in the 10 - 20 µm range). Another important factor is that many epoxies will dissolve the sulfur while curing, thus you need to experiment to determine which ones are suitable to hold your sulfur mount. I have found one or two that are suitable for our needs. Superglue can also be used. I haven't provided all the details of our sulfur embedding technique here and there are more pitfalls than I have described. The technique is difficult and somewhat unpredictable, but if everything goes right you can end up with nice microtomed sections free of any embedding material. This is useful for observing or measuring carbon by EDX if you mount your sample on a TEM grid with a SiO film (you have to watch out for carbon contamination in the microscope, though). I can imagine that there are other, perhaps better ways to mount materials in sulfur than I have described; this is just a technique that has evolved over the last several years for us. You can sublimate the sulfur by placing your TEM grid into a vacuum (roughing pump range is OK) for a few minutes, room temperature is fine. Another possible way to remove the sulfur is to gently heat your sample at atmosphere for an hour or so. Of course, if temperature sensitive organic compounds are present you might choose the vacuum method instead. Many of the IDPs that we process have very high porosities. Surface tension wicks the liquid sulfur inside the voids which simply goes away during sublimation leaving a clean section, at least in theory. You need to watch for contamination that is present in the sulfur to begin with though—especially organic contaminants. I would recommend that you purchase the very cleanest sulfur you can get and then gently distill it onto a watch glass or Petri dish that you keep covered. Dave Joswiak <joswiak@astro.washington.edu>