MICROSCOPY 101

We appreciate the response to this publication feature - and welcome all contributions. Contributions may be sent to Phil Oshel, our Technical Editor at:

Mr. Phil Oshel` (608)833-2885
PO Box 620068 eMail: oshel@terracom.net
Middleton WI 53562

Bands in SEM Images

We used to get banding in the images of our Hitachi S-800 FE-SEM, in both backscattered and secondary electron photographed images. This drove us nuts for many long months! Finally I realized that it really *only* appeared on the recording CRT, and not on the visual CRTs. I hauled out a really old SEM manual* that dealt with all kinds of weirdness. It showed banding on a CRT that was a result of dirty or deficient high voltage to the CRT. I gingerly cleaned the high voltage contacts to the CRT and the problem disappeared. It reappears every few years. and I imagine it's our humid and somewhat volcanic air that corrodes these contacts. Cleaning them up works. Hitachi remains dubious about this, by the way.

The high voltage supply to the CRT is 10 kV! Be careful! The cleaning I have done has so far not involved actually polishing contacts, but merely blowing compressed clean, dry air over the contacts, making sure they are seated, cleaning the surrounding areas, and carefully closing everything up again. There are a number of circuits in most instruments that are still energized or retain a charge when the instrument is off or even disconnected.

If the bands appear on digital images, then the source of the signal still needs to be tracked down and examined for weak contacts.

* Combing the manual for clues, I decided it may have been put out by JEOL. The spellings are British, it describes itself as a "booklet", and it is in 8" x 10" format. The terminology and references to parts of the instrument make it seem to be from *ca.* 1980? Maybe before, or as late as 1985. It's entitled *A Guide to Scanning Microscope Observation.* It has some great tips for figuring out what's going on based on what kind of image one gets. I've never seen another book that addresses problems from such a basic, practical viewpoint. Most books are full of schema and formulae.

My copy begins on page 2 and has no cover. If anyone recognizes this book, please send the complete reference to the *Microscopy Today* Technical Editor [Philip Oshel, oshel@terracom.net, or post the information on the microscopy list server.

Tina (Weatherby) Carvalho, University of Hawaii at Manoa

A SEM Tip for Examining Mixtures of Mineral and Organic Particles

The usual practice is to mount the sample in a quick-setting epoxy resin, with most of the sample in the bottom. I use silicon rubber cups with a one inch inside diameter, about 1/2 inch deep. Mix particles and resin together, about 50/50, put that in the bottom of the cup, then fill with clear resin. The set resin is then ground on a graded series of sandpaper disks with water running on them, then polished on 5 micron diamond suspension to cross-section the particles. The sample is then gold or carbon coated.

Organic or polymer particles and minerals are easily separated by backscattered imaging (BSE). The organics, being composed of light elements, will show darker than the mineral, which is composed of heavier elements. I usually use a 20 kV e-beam for BSE imaging, then vary the condenser lens to adjust the contrast and illuminate the phases I'm interested in. You can also try adjusting the kV of the beam to light up the phases of interest. A photo of secondary electrons and BSE of the same area is often helpful.

Particle size measurements may be better on a sample of grains sprinkled on a sticky tab and imaged by BSE, but topographical interference, *i.e.*, shadows, may degrade the analysis.

Mary Mager, University of British Columbia

Some Sources of Flexible Needles for Handling Microscopy Specimens

As an electron microscopist who has come from a neurophysiology background, I have used various fine needles for "dusting" off debris. You need to find the one that feels right.

Cat whiskers are long, pointy, strong, and flexible. They are particularly good for chasing tiny bubbles out of microelectrodes or capillary tubes.

Finely drawn glass: Heat a pipette or rod over a Bunsen burner and draw it out until it breaks. Find somewhere along the long string that has the right size and flexibility, but won't break and leave more debris!

Cactus spines: They come in many shapes and sizes. Also useful for pinning down things for dissection that can't come into contact with metal.

Find someone in neurobiology who does microelectrode recording and get them to make some electrodes, which are capillary tubes drawn to a very fine point. You can probably get some in the micron range. Beveled, even!

Insect Minuten pins mounted on a stick are very strong, but may scratch your substrate. They can be ground down for a finer point.

Eyelashes, beard hair, and other body hairs each have different properties. Have fun experimenting.

Tina (Weatherby) Carvaiho, University of Hawaii at Manoa

Supravital Stain for blood.

Paul Ehrlich pioneered the air-dried blood smear before the turn of the century. Air-dried, because wet fixatives washed the specimen off of the slide. Various stains were tried at first, but eventually the Romanowski stains (Wright's Giemsa, etc.) prevailed. When a smear is air-dried, the spherical white cells flatten out to become discs, introducing a number of artifacts, notably loss of nucleolar staining.

For some artifactual reason, nucleolar staining is retained in many leukemic cells, leading to the term "blast". However, with wet fixation or with supravital staining of live cells, even normal, mature small lymphocytes contain nucleoli.

So information obtainable from blood staining depends more on the mode of fixation than on the stain

Wet fixation of a blood clot is almost useless because of the overwhelming preponderance of red blood cells. Wet fixation of a buffy coat is possible if it is treated as a cell block, but centrifugation introduces a few relatively minor artifacts of its own. Any specimen that is embedded in paraffin undergoes considerable shrinkage during alcoholic dehydration.

Supravital staining means putting a small drop of blood on a stained slide

and adding a coverslip, then waiting for a few minutes for the living white cells to take up the stain. Unfortunately these slides are not permanent, although their significant information can be archived photographically.

I have not tested it in cell culture, but toluidine blue makes a great supravital stain for blood and other body fluids. I use slides dipped in 0.25 to 0.5% toluidine blue (in absolute ethanol) and then dried on end at 60° C. They keep for months. After a few minutes, a complete cell differential can be done. Each kind of granulocyte is readily identified, and the basophils are often spectacular, with bright, purple granules. Mature monocytes usually have an elongated nucleus with a tiny nucleolus at each end, and small lymphocytes have either 1 or 2 somewhat purple nucleoli. Large lymphocytes have more than one nucleolus, up to 5 or 6.

1) Dissolve 0.25-0.50% toluidine blue in 100% ethanol.
2) Warm clean slides at 60° C, dip in the alcoholic stain solution, and stand on end to drain and dry. Slides are good for months.
3) Put on a *small* drop of fluid, add coverslip, and wait 2 to 5 minutes to allow for stain uptake. Preparation is not permanent because the cells are destroyed after 20-30 minutes. They are photogenic, though, if a permanent record is desired.

This method may well work on cells in tissue culture. See Millikin, P.D. 1974. "A Supravital Stain for Nucleoli in Human Lymphocytes," American Journal of Clinical Pathology 62:520-24.

Incidentally, when this stain is used for pleural or peritoneal fluid, any cancer cells may appear spectacularly large because they are not shrunken by fixation or other processing.

In the final analysis, the stain you use depends on the information you want.

Paul Millikin, Peoria, IL

Curing a False Engagement Problem in an AFM.

I have had trouble with very high deflection increase close (microns) to the specimen surface, increasing the deflection voltage by many tens of volts, causing a false engagement. One way to obtain a true engagement is by successive adjustments of the photodiode detector, but this is a tedious exercise that does not remove the large repulsive force.

The best reason that I have come up with as to the source of the problem is from long range electrostatic charge on the surface, the tip, or both. The reason (it is believed) that the repulsive force has electrostatic origins is that touching a grounded wire to the sample puck (if mounted on a metal puck), and/or a metal component of the tip holder that is connected to the probe, often (but not always) removes the repulsive force.

Specifically when using a DI fluid cell, I have found it works well to gently touch the ground wire to the wire tip clamp that protrudes from the top of the cell. Grounding the sample and tip does not always work, possibly due to bad connections. Another method that also works well, but requires a bit of time, is to mount a Polonium source (purchased from Structure Probe, but other microscopy suppliers carry them) near the sample cell. We tape the source to the side of the AFM head, and wait for the vertical deflection to reach a constant value and re-engage.

Jason Bemis, University of Pittsburgh

Is Di-amino-benzidine (DAB) Really Carcinogenic?

Some interesting questions were recently posed on the Histonet Listserver about DAB, which is used as a marker for cells. While DAB itself has not been the subject of in-depth carcinogenicity studies, it is known to be mutagenic. Further, all members of the benzidine family that have been tested have been proved to be carcinogens. In the United States, at least, all benzidine derivatives are considered carcinogens by the NTP (National Toxicology Program), and OSHA will soon make that official.

So, the question is not about DAB *per se*, but about any substance that still retains that benzidine nucleus. None of the procedures in immunohistochemistry alter that nucleus; they merely change other parts of the molecule to make it soluble, and then later insoluble. Regardless of its physical state (powder, tablet, solution or precipitate), or the manner in which it got to that state (hydrolysis, oxidation, time), the benzidine nucleus remains. Thus it is still carcinogenic.

The only proven method of destruction of the benzidine nucleus is via acid permanganate oxidation.

Richard W. Dapson, Anatech Ltd.

TEM of Cultured Cells on Coverslips

For TEM of cultured cells, we grow the cultures on "Thermanox" tissue culture coverslips (from Nalge Nunc International, 50 sterile coverslips, 13 mm diameter is catalog 174950). The coverslips can be treated with all the same chemistry as tissue, including propylene oxide and Spurr's epoxy, which are two components which solubilize polystyrene.

Coverslips are easily processed in disposable polypropylene 50 mL centrifuge tubes, useful since the cell surface will not contact the wall of these conical tubes.

1) Sink the Thermanox coverslips cell side up in freshly made Spurr's.
2) Following polymerization, remove coverslips by first sawing a small area of the epoxy/cell/substrate, then immersing in liquid nitrogen for a few seconds and prying away the substrate. The embedded cells are now on the surface of the epoxy.
3) Re-embed two fragments of the culture face to face for cross-sections, or cut the block parallel to the face for tangential sections.

We particularly like the round 13 mm Thermanox coverslips for immunocytochemistry of cultured cells since they can be floated cell side down in a drop of 100 µL antibody - gold conjugate, which conserves reagents.

If a larger culture is desired, "Permanox" culture dishes could also be used, which are equally resistant to chemicals common in TEM processing. These are also available through most EM and other suppliers.

Douglas R. Keene, Shriners Hospital Microscopy Unit

An Easily Available Specimen for Checking an AFM tip:

The simplest commercial specimen for checking out your tip may be Nuclepore filters. These contain etched nuclear particle track pits available in standard diameters ranging from 50 nm up to well over 2 micrometers.

Our original paper on the subject is archived at Los Alamos*, and published in Ultramicroscopy 37 (1991)125-129. You can also find this and two later papers on reconstructing tip shapes in the "reference papers" section of our scanned tip and electron image web page.** Updated versions of the latter two will be made available in the archives soon as well.

* http://xxx.lanl.gov/abs/cond-mat/9712003
** http://newton.umsl.edu/stei_lab/

Phil Fraundorf, University of Missouri – St. Louis