We appreciate the response to this publication feature and welcome all contributions. Contributions may be sent to our Technical Editors: José A. Mascorro, jmascor@hotmail.com *or Phil Oshel,* oshel1pe@cmich.edu

Rhodamine Fluorescence After 15-year Storage in Methyl Salicylate

Philip L. Hertzler

Dept. of Biology, Central Michigan University

hertz1pl@cmich.edu

Fading of fluorochrome is a significant limitation to fluorescence microscopy. Several anti-fade agents, e.g. n-propyl gallate, are commonly used for glycerol-based mounting media (Longin et al., 1993; Ono et al., 2001). Samples mounted in glycerol must be kept at -20°C for long-term storage to prevent bacterial degradation. In contrast, fluorescent samples cleared and mounted in organic media can be stored indefinitely at room temperature.

Methyl salicylate or oil of wintergreen is an excellent clearing agent (refractive index = 1.53), which works well with a variety of fluorochromes. It has a pleasant aroma but is somewhat difficult to work with since it remains liquid after mounting. It was previously reported that shrimp embryos labeled with tubulin antibody and rhodamine-conjugated secondary antibody maintained their fluorescence after six months (Summers et al. 1993). These same samples, stained in November, 1990 and imaged by confocal microscopy for publication in Hertzler and Clark (1992), are still fluorescent after continuous storage in methyl salicylate at room temperature in the dark (Figure 1). The images of 62-cell stage shrimp embryos taken from these 1990 samples were collected with an Olympus Fluoview 300 laser scanning confocal microscope in January, 2006 in the Dept. of Biology, Central Michigan University. There appears to be no loss of resolution in the high magnification image (Figure 1B). Samples I have stained with Cy2, Cy3, BODIPY FL, Alexa Fluor 546, and Sytox Green and mounted in methyl salicylate have also retained fluorescence for months or years. Therefore, in addition to its excellent clearing properties, methyl salicylate is also useful for maintaining fluorescence during long-term storage of thick specimens for confocal microscopy.

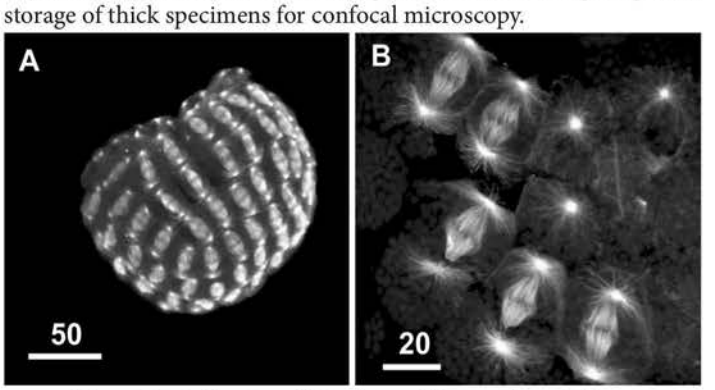


Figure 1 Optical sections of 62-cell stage shrimp embryo whole mounts labeled with E7 anti-β-tubulin antibody and rhodomine-conjugated secondary antibody. (A) Extended focus of sections 1-15 of 30 through entire embryo, step size 3 µm, using Olympus 20X 0.7 NA Plan-Apochromat objective, zoom = 3. (B) Extended focus of sections 3-5 of 10 through single cells, step size 1 µm, using Olympus 60X 1.4 NA Plan-Apochromat objective, zoom = 2. Scale bars in micrometers.

Acknowledgements:

I thank Phil Oshel, Dept. of Biology, Central Michigan University, for encouraging me to submit this observation for publication. The E7 anti-β-tubulin antibody developed by M. Klymkowsky was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

References:

1. Hertzler, P.L. and Clark, W.H., Jr. Cleavage and gastrulation in the shrimp Sicyonia ingentis: invagination is accompanied by oriented cell division, (1992) Development 116, 127-140.
2. Longin A., Souchier, C., Ffrench, M. and Bryon, P.A. Comparison of anti-fading agents used in fluorescence microscopy: image analysis and laser confocal microscopy study, (1993). J. Histochem. Cytochem. 41, 1833-1840.
3. Ono, M., Murakami, T., Kudo, A., Isshiki, M., Sawada, H., and Segawa, A. Quantitative comparison of anti-fading mounting media for confocal laser scanning microscopy, (2001) J. Histochem. Cytochem. 49, 305-312.
4. Summers, R.G., Sticker, S.A. and Cameron, R.A. Applications of confocal microscopy to studies of sea urchin embryogenesis, (1993) In: Matsumoto, B. (ed.) Methods in Cell Biology 38, 265-287.

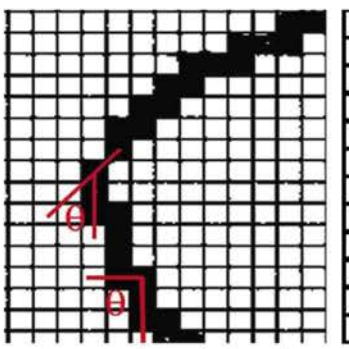
When Point To Point Is Not Enough

Carol Heckman, Marilyn Cayer, Mita Varghese

Center for Microscopy & Microanalysis, Bowling Green State University, Bowling Green, OH

heckman@bgnet.bgsu.edu

As all microscopists are taught, resolution can be measured by knowing the closest distance between two points that can be discriminated in the image. There are some occasions when we want to measure something in a digital image, and the nominal resolution isn't sufficient for the task. One of these, extracting a contour from the image, was summarized in a previous research report [1]. The principles would be valid for any filled area, such as a particle or structure from an X-ray dot map. The ground rule was set that the outermost pixel would be selected at every point on the contour. It was clear from the geometry shown in Fig. 1 that only values of 0°, 45°, 90°, 135° and 180° could be measured. Because of the stair step defect, tracing the sequence of pixel locations only gave offsets to 8 pixels, one directly below, two on the corners below, three pixels in the same location above, and one pixel on either side of the subject pixel. Therefore it was impossible to measure the true curvature of the contour.



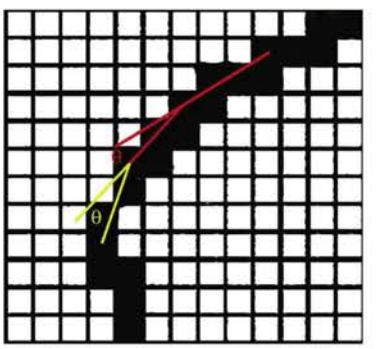


Fig. 1. 45° and 90° angles generated without dropping points on the contour.

Fig. 2. 13° (red) and 19° (yellow) angles generated after point dropping.

The solution to these difficulties was point dropping. The ideal number of points that must be dropped in order to get an accurate curvature measurement was determined [2]. The rule that two pixels be dropped for every pixel accepted gave a curvature measurement that was continuously variable between 0° and 180° (Fig. 2). Moreover the curvature calculated for an actual test figure differed from the theoretical by only 1%. The solution has been used frequently to solve biological

It's Difficult To Do A Side-By-Side Comparison When Your Coater Stands Alone.

High Resolution Sputter Coater 208HR for FE-SEM

Superior Features:

- Wide Choice of Coating Materials
- Precision Thickness Control
- Multiple Sample Stage Movements
- Variable Chamber Geometry
- Wide Range of Operating Pressures
- Compact Modern Benchtop Design

TED PELLA, INC.
Microscopy Products for Science and Industry

4595 Mountain Lakes Blvd., Redding, CA 96003-1448
Phone: 530-243-2200 or 800-237-3526 (USA) • FAX: 530-243-3761
Email: sales@tedpella.com Web Site: http://www.tedpella.com

problems where cell shape analysis is required [3]. Point dropping was encoded into BGSU's software for tracing a contour which can be run by logging onto the anonymous FTP server <elvis.bgsu.edu>

References:

1. Olson, O.C., Larson,N.M., and Heckman,C.A. (1980). Classification of cultured mammalian cells by shape analysis and pattern recognition. *Proc.Natl.Acad.Sci. USA.* 77, 1516 -1520.
2. Heckman, C.A and Jamasbi, R.J. (1999). Describing shape dynamics in transformed cells through latent factors. Experimental Cell Research.246, 69-82.
3. Heckman, C.A., Plummer, H.K., and Mukherjee, R. (2000). Enhancement of the transformed shape phenotype by microtubule inhibitors and reversal by an inhibitor combination. International Journal of Oncology. 16, 709 – 723.

Making Replicas of Surfaces for TEM and SEM

Mary Mager

Materials Engineering Department, University of British Columbia

mager@interchange.ubc.ca

Carbon Replicas

Before SEMs were invented and when they were still of relatively poor resolution, one way to see the fine details of a sample surface was to prepare an electron-transparent replica of the sample surface and view it in a TEM. The carbon-coated surface of the sample was shadowed with a heavy metal to make a replica that mimicked the topography of the original surface, in a sample that could be viewed in the TEM.

We have found some value in this old technique; to examine second-phase particles freed from the metal matrix for EDX, diffraction, and morphology studies--while preserving the original position and distribution of the particles, historically called "extraction replication."

To prepare a carbon replica:

1. Polish the specimen to a one micron diamond polish, or equivalent.
2. Lightly etch the surface with the appropriate etch for the metal. When we were using the technique for low-carbon, micro-alloyed steels, we used two percent Nital (2 ml. Nitric acid and 98 ml. methanol) for about 10 seconds to slightly etch the surface. Rinse and dry.
3. Carbon-coat the sample surface with at least 20 nm of carbon in an evaporator.
4. Score the carbon coat with a razor blade into approximately 3 mm squares.
5. Etch the specimen by immersing in a stronger etchant for one to two minutes. For the low-carbon, micro-alloyed steels, we used five percent Nital. You should see the carbon coat start to wrinkle.
6. Submerge the specimen in a dish of distilled water with the carbon-coated side up. The carbon squares should pull free and float to the top of the water. Use a plain copper TEM grid of 75 or 100 mesh to scoop up the carbon squares from below the surface of the water.
7. Blot the grids dry on filter paper, carbon side up.

You should be able to image the replicated surface of the etched material in the TEM, preserved in the carbon film. The second-phase particles that were caught and held by the carbon film should be darker and their position and morphology preserved by the carbon film. They are free of any interference from the matrix metal for EDX and electron diffraction studies.

The best source of recipes for etchants is George F. Vander Voort's book: METALLOGRAPHY Principles and Practice, McGraw-Hill Book Company.

Cellulose-Acetate Replicas

To sample the morphology of a surface that is too large to go into an SEM, or that is not available for examination or to sample the corrosion product from a surface, a cellulose-acetate replica can be made of the surface and examined in the SEM. Cellulose-acetate (CA) replicating tape and sheet is available from most EM catalogue suppliers. Cut a strip of the tape large enough to cover the area you want to sample, plus a piece at the end for a handle. I usually use about a 10 to 15 mm long strip, about 10 mm wide. Bend the last three mm up at a right angle to make a little handle.

To make a cellulose-acetate replica:

1. Place the strip of CA on a pile of filter papers soaked in acetone and leave it on there for 10 to 20 seconds until the bottom gets sticky. You will have to experiment to see how long to leave it, so the CA gets soft but does not melt away.
2. Put a puddle of acetone on the surface to be replicated. Use the handle on the CA strip to pick it up and put it on the puddle of acetone. Try to exclude any air from under the CA strip.
3. Leave the CA on the sample for at least half an hour, until the acetone is all evaporated and the CA is firm again.

Carefully peel the CA off the sample and trim off the handle. Glue it, replica side up, on an SEM stub. Carbon coat the CA for EDX analysis of any corrosion product that has been stripped off or gold coat it for morphology or fractography. It can also be viewed in variable-pressure mode uncoated. Remember that some features will be reversed, e.g. the "cup-and-comb" features of a ductile fracture will now be a series of bumps. Brittle fracture and fatigue should look the same as the real sample. You can strip multiple replicas from a corroded surface to clean the metal gently and examine the layers of corrosion product.

There are many other specimen preparation protocols using replication techniques that are useful in all fields of electron microscopy. I hope others will be willing to share their knowledge of this art in future Tech Notes.

Addendum to TJ Collins article on Mounting Medias*

Gayle Callis

Veterinary Molecular Biology

Montana State University, Bozeman MT

uvsgc@msu.oscs.montana.edu

A caveat about using fingernail polish cover slip sealing for tissues containing Green Fluorescent Protein (GFP) was given by Chalfie et al, 1994.[1] They reported the chemicals in nail polish interfered with GFP fluorescence, a problem we also experienced in our laboratory. Clontech, a GFP supplier, warns customers about this problem. The chemical in question may be the alcohol that leaches into aqueous mounting media. We dilute permanent acrylic mounting media with its solvent (e.g. xylene or toluene) so the media is thinner than the nail polish, less messy to use, and easier to apply around cover slip edges. Toluene and xylene are not miscible with water and cannot leach into aqueous mounting media although other chemicals may be involved. Diluted media dries rapidly and also prevents 'drying' retraction bubbles from forming under the cover glass with two hard set anti-fade mounting media (Vectashield, Vector Laboratories, CA and Prolong Gold Molecular Probes, OR).

* Collins TJ, *Mounting Media and Antifade Reagents*, Microsc. Today, 14, 1, January 2006.

1. Chalfie M, et al., *Green Fluorescent Protein as a Marker for Gene Expression*, Science 263:802-805, 1994.