MICROSCOPY TODAY

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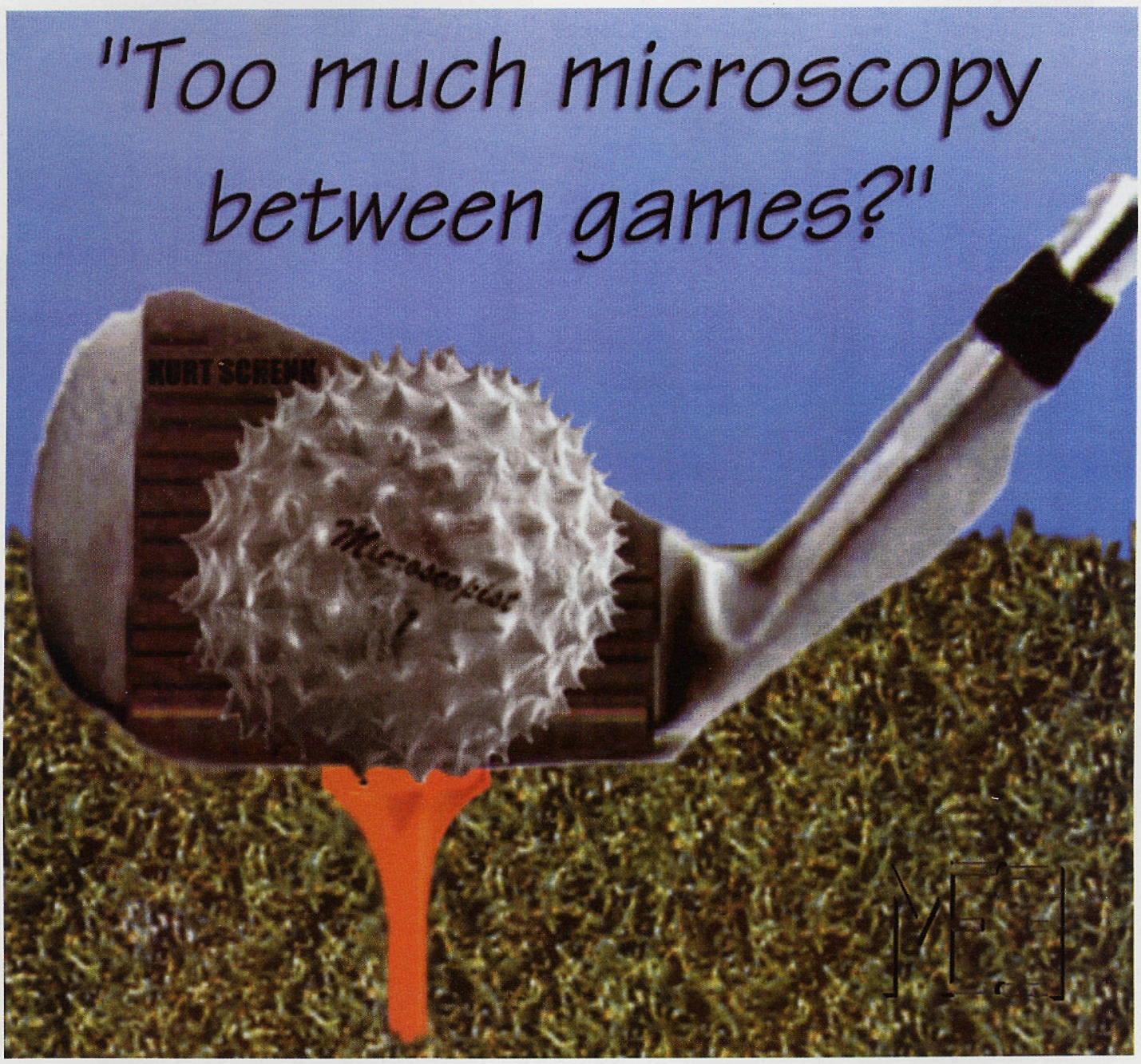

# With the New LEO VPSE the difference is clear...


Take a look at the two micrographs of pollen on the left. Both were taken in variable pressure mode, one using a backscattered electron detector, the other using LEO's new VPSE (Variable Pressure Secondary Electron) detector. For low atomic number material such as this, the difference is clear-and so is the image taken with the VPSE!

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Circle Reader Inquiry #5

## BREAKING OLD RULES

Stephen W. Carmichael,[1] Mayo Clinic

It has been dogma for more than a hundred years that the light microscope cannot resolve structures that are much closer together than the length of the wavelength of light. This limit is on the order of a few hundred nanometers. This rule was "broken" by using the electron beam as the illumination source. The shorter wavelength of the electron beam gave correspondingly better resolution, down in the nanometer range. The rule was "broken" again when non-optical scanning probe microscopes (such as the scanning tunneling and atomic force microscopes) were developed. This technology gave us resolution in the sub-nanometer range, imaging individual atoms and molecules. However, scanning probe microscopes scan the surface of the specimen, excluding what's underneath from our view. Wouldn't it be great if another rule could be "broken" so that we could look at individual molecules beneath the surface? As you have already guessed, this has been accomplished. Antoine van Oijen, Jürgen Köhler, Jan Schmidt, Michiel Müller, and Fred Brakenhoff have achieved super-resolution using visible light.[2]

They used a technique called Spectrally Selective Imaging to determine the position of single molecules within a crystal matrix. This technique takes advantage of the fact that a fluorescent molecule will behave slightly differently to a selected wavelength of light, depending on its immediate surroundings. van Oijen *et al.* used individual pentacene (an aromatic hydrocarbon) molecules embedded in p-terphenyl host crystal. For seven of these molecules, they found that the relative excitation frequency varied over a range of two to three thousand megahertz. Selecting one of these molecules they tune the excitation laser burn to achieve maximum emission from this molecule. Under cold (liquid helium) conditions, the centroid of the fluorescing molecule can be calculated to within 40 nm in the xy plane. The axial (z) position is obtained by measuring the radial size of the photon distribution as a function of the detector position along the optical axis This yielded a resolution of approximately 100 nm. This improvement in resolution represents an enhancement of about 20- and 65-fold in the xy and axial planes, respectively. This was repeated for the other molecules, each at its respective optimal excitation wavelength, resulting in a 3 dimensional map of the position of each pentacene molecule within the volume studied.

Interestingly, van Oijen *et al.* observed that the distribution of the pentacene molecules within the crystal was non-random. In three different experiments, the molecules were in a much more narrow axial portion of the crystal than would have been predicted by random distribution (within a plane of 1.3 μm rather than the expected 7 μm). They theorized that this resulted from local structural properties of the crystal matrix. On this length scale, this would be undetectable by other optical techniques

This is another demonstration of the old rules being "broken." By combining single-molecule spectroscopy with position-sensitive imaging, van Oijen *et al.* enhanced the limit of resolution using light by an order of magnitude. It was demonstrated that the method of Spectrally Selective Imaging may be used for molecules that undergo photobleaching or spectral diffusion, even if this occurs within a few seconds. It certainly seems possible to now determine the position of fluorescent molecules within structures other than a crystal, possibly within a cell, or within the nucleus. It will be exciting to see how this breakthrough will be utilized to map the position of molecules with greater precision than has ever been possible!

1. The author gratefully acknowledges Dr. Jürgen Köhler for reviewing this article.

2. Van Oijen, A.M., J. Köhler, J. Schmidt, M. Müller, and G.J. Brakenhoff, 3-Dimensional super-resolution by spectrally selective imaging, *Chemical Physics Letters* 292:183-187, 1998. See also van den Berg, R., Molecular imaging beats limits of light, *Science* 281:629, 1998.

## Front Page Image
## Third Prize - Just For Fun Micrograph Contest

At the recent Microscopy & Microanalysis '98 Conference in Atlanta, Microscopy Today held a "For Fun" contest - with micrograph entries being a composite of two or more images, at least one being microscopical in nature. With fourteen entries and hundreds of "votes", Kurt Schenk, from Material Evaluation and Engineering, Inc. won the third prize. His description of this third prize image is, "This image is a reflection of one possible result from too much microscopy. The "Golf Ball" is actually an ambrosia (ragweed) pollen grain imaged using a scanning electron microscope".

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THERMOMICROSCOPES

FROM THE OFFICE OF THE PRESIDENT

# An open letter to microscopists:

## THE WORLD'S LARGEST

I am pleased to announce that ThermoSpectra has completed the purchase of scanning probe microscope pioneer TopoMetrix Corporation and merged it with Park Scientific Instruments. The combined firm, THERMOMICROSCOPES, is part of Thermo Instruments, the world's largest instrument company. THERMOMICROSCOPES joins over 150 companies in the Thermo Electron family providing worldwide technical and product support you can always count on.

## TECHNICAL INNOVATION

THERMOMICROSCOPES is a company representing much more than the sum of its parts. The extent of its SPM intellectual property and patent portfolio is second only to that of IBM. The combination of Park Scientific Instruments' extensive range of advanced research products with TopoMetrix' industrial products – such as those in wide use in the optical data storage, polymer research, materials analysis and electronics testing – is the most complete in the SPM industry. With many hundreds of satisfied customers in Fortune 500 companies, academia and government agencies throughout the world, THERMOMICROSCOPES instruments are well recognized as innovative, leading-edge products.

## HONESTY AND INTEGRITY

To you, a professional involved in the exciting world of microscopy, this is a major step. You will be dealing with an honest, world-renowned instrument company. It combines large company resources with the enthusiasm, culture and entrepreneurial atmosphere of a small company. It offers the best of research and industrial experience, as well as an open and honest approach in all customer dealings. But it is the established product advantages that set THERMOMICROSCOPES even further apart from the competition: features like open architecture in both software and hardware; the industry's finest optics; upward and downward compatibility wherever possible; and software with the highest functionality. These are advantages you will get only from THERMOMICROSCOPES, a company with the honesty, integrity and worldwide resources necessary to stand behind its products and support you as your future requirements evolve.

## AN EXCITING FUTURE

You will be hearing and reading a great deal about THERMOMICROSCOPES in the days to come. In the meantime, I invite you to visit our web page at www.thermomicro.com for up-to-date developments and product information. Or feel free to call, write or e-mail me, if you have any questions about THERMOMICROSCOPES and how we can directly assist you in your work.

Sincerely,

Gary D. Aden

Gary Aden, President
THERMOMICROSCOPES

1171 BORREGAS AVENUE, SUNNYVALE, CA 94089 USA TELEPHONE 408.747.1600 FAX 408.747.1601 WEB WWW.THERMOMICROSCOPES.COM

## KEVEX AND NORAN ARE BECOMING ONE!

Thermo Spectra recently announced the restructuring of Kevex Instruments, including both their microanalysis and XRF product lines.

The Kevex microanalysis efforts, including both products and personnel, will be integrated within NORAN Instruments. A stated objective is to have a stronger product offering to the EM market and to provide more choices to customers. The Kevex microanalysis products, including the Sigma product line, will be supported by the new organization.

The Kevex XRF system line will be integrated within Spectrace Instruments. The objective of this new organization will be to build a new XRF/XRD market focused business.

The support for the products of both companies will continue without disruption. All customers should continue to contact their previous organization for support.

## TOPOMETRICS AND PARK SCIENTIFIC ARE BECOMING ONE

Refer to the letter by Dr. Gary Aden on the preceding page.

## New Micro-Technology Division

Ladd Research Industries has announced the formation of a new micro-technologies division which will specialize in advanced aperture and micro-hole development.

This new micro technology division also produces advanced apertures/slits for a wide array of applications including x-ray collimators, FIBs, and other beam and flow control devices. Currently three communications satellites equipped with Ladd control devices are now in geo-synchronous orbit above the earth.

## 1999 Microscopy Society of America Awards

All MSA Members are reminded that applications are currently being solicited for the 1999 MSA Awards. The Awards include:

**MSA Distinguished Scientist (Biological Sciences)**
**MSA Distinguished Scientist (Physical Sciences)**
**MSA Burton Metal**
**MSA Outstanding Technologist (Biological Sciences)**
**MSA Outstanding Technologist (Physical Sciences)**
**MSA Morton D. Maser Distinguished Service Award**

The deadline for receipt of Awards Nomination Packages is December 31, 1998. Please contact Gracie Burke (mgburke@pitt.edu) or the MSA Business Office for additional information

American Chemical Society is once again offering "Analytical Light Microscopy for Chemists", a total immersion, hands-on course, in conjunction with PITTCON '99, March 5-7, 1999, in Orlando, FL. Curriculum details are available from the course organizer at www.MME-Microscopy.com/education and registration detail from ACS (800-227-5558), www.acs.org

## Microwave Cryofixation

Lee van Hook
Piltdown Research Institute, Munchausen University

There is much discussion on the best way to fix biological specimens, often advocating cryofixation or microwave fixation. These discussions sometimes become acrimonious, and we at the P.R.I. fear the eventual division of microscopists into rival lab gangs, the "Waves" and the "Cryos".

Wishing to forestall this, and because Leonard Bernstein is no longer with us to write *West Lab Story*, we have been working on a method of combining the two fixation methods. Less romantic than rival gang members falling in love perhaps, but more practical.

The basic premise is this: microwave radiation is obviously periodic, as is the motion of molecules. Heating occurs when microwaves of a specific frequency interact in some manner with the motions of particular molecules, the frequency determining with which molecules (or bonds) the microwaves interact. The physics of this are poorly understood, but since both the microwaves and the molecular motions are periodic phenomena, it may be assumed that absorption of microwave energy is strongest when the microwave frequency resonates with the target molecule's motion. The microwave therefore stimulates the molecule's motion, and so heating occurs.

Well then, if this is so, what happens with the microwave is 180° out of phase with the target molecule's motion? The microwave ought to cancel the molecule's motions, the way out-of-phase sound waves cancel out each other (something well known to those who have bought noise-cancelling headphones and are no longer subjected to crying-baby syndrome on long plane flights). This cancellation quiets the molecule's motions, resulting in cooling instead of heating. Fantastic as this may sound, it is no stranger than bombarding fast moving atoms with lasers to slow (that is, cool) the atoms to near absolute zero.

The obvious objection is that the molecular motions are random, and need to be synchronous to be cooled by the out-of-phase microwaves. The same argument applies to heating, since the heating results from the interaction of the microwaves and molecules and isn't just an increase in the kinetic energy of moving molecules or atoms (as when water is boiled on a stone). In the usual case, random interaction leads to increased friction, and therefore heating. If random interaction leads to *decreased* friction or motion in the majority of molecules, we get cooling.

Regular microwave processors use a water load to help balance the power in the oven, and keep the heating even. There is a need for analog of this in cryofixation, and we have found frozen daiquiris excellent for this purpose.

The great advantage to microwave cryofixation is much the same as for microwave heating: the temperature of the specimen is affected throughout its volume. This means that larger specimens can be cryofixed than before. Also, with properly tuned microwaves, very rapid cooling rates can be achieved, so amorphous freezing occurs deep in the specimen, and not just In a 10 or l00 µm surface layer.

How exactly the microwaves interact with the specimen to achieve this freezing is of course proprietary information (OK, we don't understand it either), but readers can expect the P.R.I. line of CryoWave®™® ovens to be on the market as soon as our technology-transfer division, Oxymoron Inc., is in business.

## COMING EVENTS

**McCrone Research Institute (Selected) Microscopy Courses,** Chicago, IL
Nov 9/13 '98: Particle Isolation, Manipulation and Mounting for Additional Analysis
Nov 30/Dec 4 '98: Scanning Electron Microscopy
March 1/5 '99: Conoscopic Methods
April 12/16 '99: An Introduction to TEM – Techniques and Applications
April 12/16 '99: Advanced FTIR Microscopy
June 21/25 '99: Electronic Image Acquisition, Processing & Analysis
Nancy Daerr: (312)842-7100, Fax: (312)842-1078, ndaerr@mcri.org

✓ Nov 2/3 '98: **Image Analysis Workshop** (Univ. of Maryland) College Park, MD. Dr. John Russ: (301)495-5964, email: http://www.mediacy.com

✓ Nov 2/6 '98: **45th International Symposium Covering Thin Films, Microelectronics, Nanostructures, Processing, Surfaces, and Vacuum** (AVS), Baltimore, MD. AVS: (212)248-0200, avsnyc@vacuum.org

✓ Nov 15/20 '98: **37th Eastern Analytical Symposium and Exposition.** Somerset, NJ. (302)738-6218, Fax: (302)738-5275, Email: easinfo@aol.com

✓ Nov 11 '98: **28th Scottish Microscopy Group Symposium.** University of Dundee: http://www.abdn.ac.uk/~nhi691/smg98.htm

✓ Nov 15/20 '98: **1998 Eastern Analytical Symposium.** Somerset, NJ, (302)738-6218, easinfo@aol.com

✓ Nov 16/20 '98: **Scanning Electron Microscopy & X-ray Microanalysis** (SUNY), New Paltz, NY. Dr. A.V. Patsis, (914)255-0757, Fax: (914)255-0978, email: inquiries@ims-np.org

✓ March 7/12 '99: **PITTCON '99.** Orlando, FL (412)825-3220, Fax: (412) 825-3224, email: expo@pittcon.org

✓ March 22/25 '99: **11th International Conference on Microscopy of Semiconducting Materials.** (RMS & MRS) University of Oxford. http://www.iop.org/Confs

✓ April 11/14 '99: **SCANNING '99:** (FAMS, Inc.) Chicago, IL, Mary K. Sullivan: (201)818-1010, Fax: (201)818-0086, fams@holonet.net

✓ April 11/15 '99: **FOCUS ON MICROSCOPY 1999 – 12th International Conference on 3D Image Processing in Microscopy & 11th Internation Conference on Confocal Microscopy** (European Molecular Biology Laboratory). Heidelberg, Germany http://www.embl-heidelberg.de/Conferences/FocusOnMicroscopy

✓ **LEHIGH MICROSCOPY SCHOOL,** Bethlehem, PA
June 14/18 '99: SEM and X-ray Microanalysis
June 13 '99: Introduction to SEM and EDS
June 21/25 '99: Advanced Scanning Electron Microscopy
June 21/25 '99: Quantitative X-ray Microanalysis
June 21/24 '99: Analytical Electron Microscopy
June 22/25 '99: Atomic Force Microscopy
June 21/24 '99: Microdiffraction
Info: Ms Sharon Coe: (610)758-5133, email: slc6@lehigh.edu

✓ June 16/27 '99: **3D Microscopy of Living Cells** & June 29/July1 '99: **3D Image Processing Workshop** (Univ of British Columbia) Vancouver, BC, Canada. Prof. James Pawley: (608)263-3147, jbpawley@facstaff.wisc.edu

✓ June 21/25 '99: **15th Annual Short Course on Molecular Microspectroscopy** (Miami Universtiy) Oxford, OH (513)529-2874, fax: (513)529-7284, email: http://www.muohio.edu/~sommeraj

✓ Aug 1/5 '99: **MICROSCOPY & MICROANALYSIS '99** (MSA) Portland, OR

✓ April 11/13 '00: **MICRO 2000** (Royal Microscopical Society) London

✓ June 26/30 '00: **7th Asia-Pacific Conference on Electron Microscopy** Singapore. eMail: micngml@nus.edu.sg or medlab2@nus.edu.sg http://www.med.nus.edu.sg/micsoc/7apem

✓ July 9/14 '00. **12th European Congress on Electron Microscopy.** Bruno, Czech Republic. http://www.eurem2000.isibrno.cz/

## Quantitation In Immunohistochemistry

Barry R. J. Rittman, The University Of Texas

Valuable information concerning the relative amounts of the end proucts of histochemical and immunochemical reactions present in sections may be provided by qualitative evaluations, however, greater reliance is often placed on quantitative evaluations. Many quantitative evaluations are based on the use of image analysis and optical density readings of the visible end products. An important question is whether these quantitative measurements are reliable, accurate and reproducible, and if quantitation of these reactions offers any real advantage over qualitative evaluations.

"Intensiy" and "optical density" are terms, which are often used to describe the amount of the end product of a reaction in a section, but these terms are often used in an undefined and indiscriminate manner. **In optics**, intensity of light refers to the amount of the light that is available to form the image. This measurement can be applied to both transmitted and reflected light microscopy and **is measured on a linear scale**. On the other hand, in reference to staining and histochemical reactions, intensity is used to **subjectively** describe the relative amount of the end product that is present (*e. g.*, - to ++++).

**Optical density** is the amount of light that passes through an object compared to the total amount of light incident to that object. Dark objects, which impede transmission of light, therefore, have a high optical density and lighter objects a lower optical density. **Optical density is measured on a logarithmic scale** from 1 (the lightest) to 4 (the darkest).

To illustrate some of the problems in measuring amounts of the end product, let us assume that tissues have been subjected to a **standardized** procedure of fixation in buffered formalin and paraffin wax processing. The histochemical procedure has resulted in a colored end product. Seven µm thick sections from different blocks of tissue have been measured on a video digital image analyzer. The optical density measurement from a section of the first block of tissue was 0.9 and from the second tissue was 1.0. As optical density is on a logarithmic scale, these measurements seem to indicate that, based on the limited number of sections examined, one tissue has twice the amount of material than the second.

In determining the reliability to be placed on this data, at the very least the following criteria should be applied:

1. Are the sections of the same thickness?
2. Was a standardized method of preparation used for each of the slides?
3. Were the measurements from comparable areas in different sections? Made by the same operator? Reproducible?
4. Do measurements correlate to the ratings the pathologist would give with a subjective or semi-quantitative method of evaluation? As a mini mum, are positive and negatives strictly comparable?
5. Were appropriate negative and positive controls used?
6. Does the counterstain obscure some of the weaker reactions?
7. Is the relationship between the color developed and the amount of substrate or antigen present known?
8. Can any artifacts present be identified?
9. Is the same image analysis system with the same settings used for all measurements?

In general, sequential sections cut from the same block at a particular setting are regarded as having plano-parallel upper and lower surfaces and to be of uniform thickness. There is, however, considerable compression during cutting and some non-uniform expansion between different tissue components during the section mounting. Compression of paraffin sections is dependent on a variety of factors including section thickness, size of the block, degree of hardness of tissue and its composition and many other technical factors. All other factors being equal, compression is usually inversely proportional to the section thickness. What is the thickness of the sections being measured? Direct measurement of section thickness is rarely carried out as this is cumbersome and time consuming; however if one section is 3 microns and the other is 4 microns then there is a 33% difference in the volume of tissue between these two sections. Careful measurements have shown that many sections are wedge shaped rather than plano-parallel. While variations in thickness between sections do not appear to be large, errors in measurement are usually cumulative and this discrepancy may be significant especially at higher magnifications.

Controls to decrease or eliminate these discrepancies can be incorporated. Controls can take a variety of forms, *e.g.*, a uniform block with a known concentration of protein such as gelatin or a relatively uniform tissue such as liver. Ideally, a block of this control material should be subjected to the **identical** procedures of fixation, processing, cutting, mounting, and staining as the tissue under investigation and also embedded in the **same block**. The reaction product in this standard control can then be directly related back to previous measurements. With careful planning this standard can also be the positive control.

Areas of the tissue, which do not appear to be stained, are routinely used as negative controls (background) to set a zero reading for the measurement of optical density. Significant differences can, however, exist between different "background" areas in the same section.

Operator measurement error can be significant and must be minimized and standardized. It is important to determine the error that is present for each individual operator and its repeatability. Initially, operator error can be determined by measuring single sections several times and calculating the standard error. It is usually not possible to produce identical readings, however, readings should be within well-defined limits. The acceptable degree of error will depend, to a large extent, on the differences being measured. The smaller the differences between samples, the more critical a low operator error becomes. As samples are often measured at different times, it is also important to determine reproducibility of measurements for the same sections at different times. It is critical that appropriate positive and negative controls be used.

The image analysis system should also be calibrated to reflect the expertise of the individual pathologist. Slides that can be easily ranked by a pathologist *e.g.*,on a scale of 1 through 5 should be measured and the readings compared to the pathologist's ranking levels. This will be a guide in setting the minimum and maximum levels of acceptance. If more than one pathologist is involved then each pathologist needs to be individually calibrated to the same standards.

A counterstain in a contrasting color may be useful for orientation but should not obscure weak positive reactions or be difficult or impossible to eliminate from the measurements. For orientation purposes, an alternate to the use of a counterstain is to consider a different type of microscopy such as darkfield, phase contrast, modulation contrast or interference microscopy to provide the additional contrast necessary.

Standardization of the reaction with attention to reagents, dilutions, expiration dates, times in reagents and the elapsed time before measurements are taken is important to varying degrees. There may be difficulties if the reaction relies on an individual technician to determine the final appropriate intensity of the end product. If either inadequate controls or no controls are used for calibration then additional problems will occur.

Several artifacts are possible during preparation of the stained section. For example, if the section contains areas with folds, undulations or bubbles, the reaction may have proceeded from both sides of the section, resulting in a greater intensity of staining in those areas. These areas may be in specific locations suggesting a greater amount of end product.

It is important to use the same image analysis system with the same illumination and filters to standardize measurements. If color is the major factor used in measurements then the color temperature of the illumination (and film if used) is an important factor. The illumination must be set at the appropriate level for this color temperature. If the intensity of light is too great for the image analysis input or film then this can be decreased using neutral density filters which will decrease intensity without altering the color temperature.

In addition to the above criteria, if photographic prints or slide transparencies are used as the image input they must be standardized. Daylight film can be substituted for tungsten film if the appropriate filter is used to adjust the color temperature. Color balance will, however, vary between film types from different manufacturers and even within batches of the same film, especially if stored at room temperature. For a more uniform result it is advisable to purchase a large batch of film and store in the refrigerator or the minus 20 freezer.

Many photographic processing companies adjust film processing to the color balance they consider appropriate and this is more apparent with color print than color slide film. Standardization requires that a standard sample of a color print or color slide, as applicable, be supplied each time a film is submitted to ensure that the photographic processing is uniform from film to film.

The above considerations do not necessarily take into account other factors that are important when using fluorescent markers, *in situ* hybridization and autoradiography. Fluorescent markers have additional considerations of fading, standardization of reagents and procedures.

Procedures which measure exposed photographic emulsion require the use of standards such as aluminum step wedges or sections of known radioisotope content on the same slide. Controls on separate slides will create additional errors.

With the above points in mind the question is, "Do optical density measurements provide useful quantitative data from slides stained for immunohistochemistry or histochemistry when compared to subjective (qualitative) evaluations? At first glance the comments above might suggest that we are dealing with an insurmountable problem with so many potential errors that not all can be recognized, eliminated or standardized. The keys to successful measurements are standardization and controls.

An image analysis system, if correctly calibrated, operated by a skilled technician and using appropriate controls, can provide quantitative data that is both consistent and meaningful. To reach that point however requires expense, training and meticulous attention to detail. The critical point in the entire process is the definition by the pathologist of clear parameters, including examples of positive and negative cases, to ensure that measurements within a specific range level reflect the pathologist's perception of the diagnoses. In cases, which are unequivocally positive or negative, measurements may offer no real advantage to the pathologist. In other instances, which could be classified as marginal, the data may be a deciding factor, providing that the appropriate levels of acceptance have been clearly defined.

The skill of an experienced pathologist will never be replaced by an image analyzer, however, the quantitative data provided by such systems may aid in the clarification of difficult cases and enable more specimens to be examined in a shorter time period. ■

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# Artifacts and Their Sources*

McCrone Research Institute

Artifacts in preparations are sample contaminants and may constitute a nuisance if they are not recognized, or an error if they are mistaken as part of the sample. Most artifacts have two characteristics: they are common substances and they are usually minor or trace components of the sample. Occasionally, artifacts are found as a major part of what is assumed to be the sample; for example, microscopical analysis of a plugged pneumatic valve showed cornstarch as the major component. It was soon discovered that the valve had been sent in a plastic bag dusted with cornstarch. Only one percent of the particles were actual sample.

Samples can be contaminated with artifacts during collection and during slide preparation. Contaminants that occur during sample collection are the most difficult to categorize as artifacts, especially if their sampling history or source is unknown. Fortunately, the composition of particles from clean rooms, stacks, process lines and products can be predicted, so the artifacts should be outstanding.

Artifacts that occur during sampling are nearly limitless. Some common ones are metal pieces and filings from instrument threads, valves, etc., and fragments from tools used to scrape, pry or abrade the sample. Particles collected on membrane filters are occasionally removed by dissolving the filter and "freeing" the particles by repeated centrifuging and solvent washing. Consequently, a small amount of membrane filter may be found in the dried sample residue. The dry sample substrate or container can also contribute artifacts. In rough decreasing order of the quantities of particles they contribute, facial tissue, filter paper, glass fiber filters, transparent tape, glass vials, plastic bags, membrane filters and plastic vials can all contaminate samples.

The microscopist and his environment contribute most artifacts. The microscopist's clothes, skin and hair generate hundreds of thousands of particles. Fragments from the cover glass, fibers from lens tissue or facial tissue used to clean the slide and cover glass, and general laboratory dust are also prime artifacts. The microscopist can, however, control or monitor these artifacts. The best control is a clean bench with a laminar flow of HEPA-filtered air (rated to remove 99.97% of 0.3 µm and larger particles), though this preventive measure is costly. It is cheaper to identify or at least recognize, particles in the sample work area. This can be done by sampling the lab dustfall by exposing a clean slide. This slide can then serve as a permanent standard of that area's artifacts. From time to time, similar standards should be prepared to account for seasonal artifacts such as pollen, insect parts, oil soot, etc.

An alternate procedure is to keep handy a photographic reference of common artifacts, such as the accompanying set of figures. As an additional aid, the captions contain a very brief description of the artifacts' prominent features. These and hundreds of other particles are fully described in The Particle Atlas[2]. An atlas of artifacts found in histological sections is also available[3]. All of the photomicrographs here were prepared in the Aroclor *5442* mounting media (refractive index = 1.66) and photographed with slightly uncrossed polars. ■

*Adapted from an article originally published in The Particle Analyst in 1968, available from McCrone Research Institute (1).

(1) McCrone Research institute, 2820 South Michigan Avenue, Chicago, IL 60616-3292. VOICE (312) 842-7100; FAX (312) 842-1078; www.mcri.org.

(2) The Particle Atlas, Electronic Edition, is available on CD-ROM from McCrone Research Institute, from McCrone Accessories and Components, 850 Pasquinelli Drive, Westmont, IL 60559, or from McCrone Scientific Ltd, 73 Maygrove Road, London NW6 2BP.

(3) An Atlas of Artifacts Encountered in the Preparation of Microscopic Tissue Sections. Samuel Wesley Thompson, D.V.M., M.S. and Lee G. Luna, D.Lit., H.T. (ASCP); Charles C. Thomas Publisher, Springfield, IL (1978), 190 pgs., illus. (500 photomicrographs).

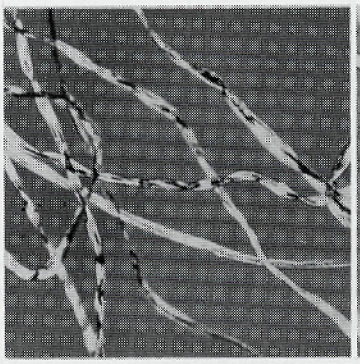

Figure 1 Cotton 250X

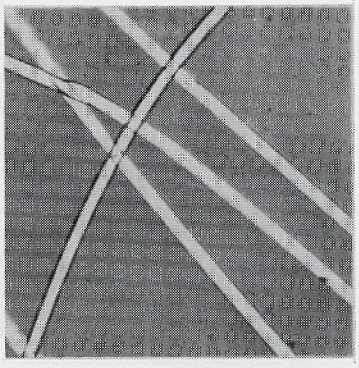

Figure 2 Nylon 250X

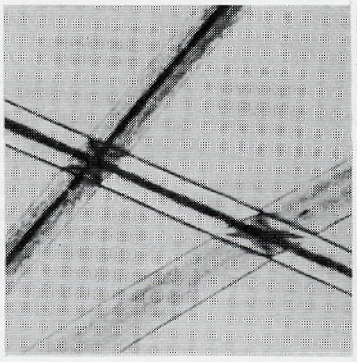

Figure 3 Human hair 250X

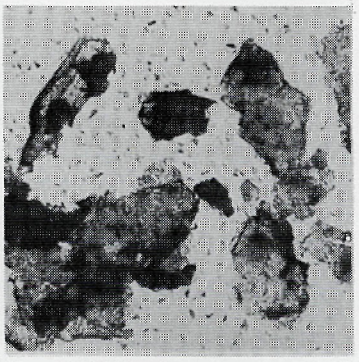

Figure 4 Epithelial cells 100X

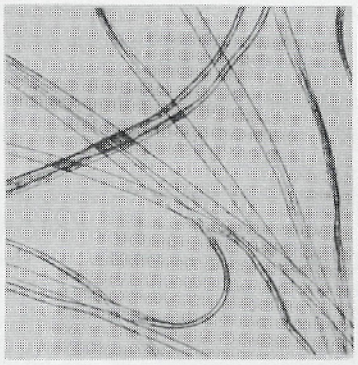

Figure 5 Orlon 250X

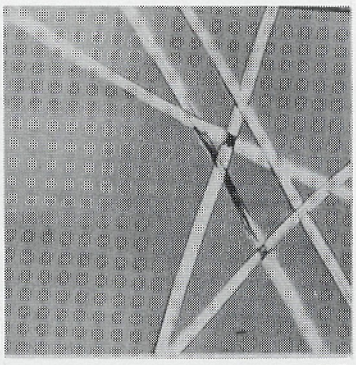

Figure 6 Viscose rayon 250X

Figure 1. *Cotton fibers* are colorless or dyed, birefringent ribbons, generally twisted, resembling slightly wound rubber bands. Their refractive indices are less than the refractive index of the medium. Extinction is seen, if at all, as a traveling black band as the microscope stage is rotated. The most common sources of cotton fibers are personal clothing and lab coats.

Figure 2. *Nylon fibers* are smooth, colorless, transparent fibers with high birefringence for a fiber (0.060). These have a uniformly round crossection. The refractive indices are less than the refractive index of the medium. These fibers are primarily generated from fabrics.

Figure 3. *Human hair* has a scale pattern not easily seen in this mounting medium. The central canal, or lumen, if seen, appears dark and may be continuous or fragmented Some fibers are so heavily pigmented that they are nearly opaque. The refractive indices are less than the refractive index of the medium. Dandruff flakes, paper fibers and other debris are often attached to the fibers.

Figure 4. *Epithelial cells* are colorless, transparent flakes. Aggregates are light tan sheets (dandruff). They are isotropic but occasionally appear weakly birefringent. The refractive index is below 1.66.

Figure 5. *Orion fibers* have a uniform diameter and are often dumbbell-shaped in cross section, giving the appearance of having a central canal or a bright line through the center. The fibers are usually delustered with titanium dioxide and appear pigmented. These textile fibers are weakly birefringent, having indices less than 1.66 and a negative sign of elongation.

Figure 6. *Viscose rayon* is uniform and transparent, with a convoluted cross section making the surface appear striated. The birefringent fiber has indices below 1.66, and usually shows first- and second-order polarization colors.

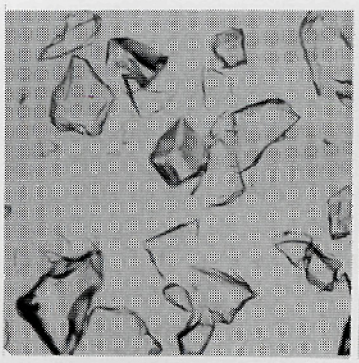

Figure 7 Glass fragments 250X

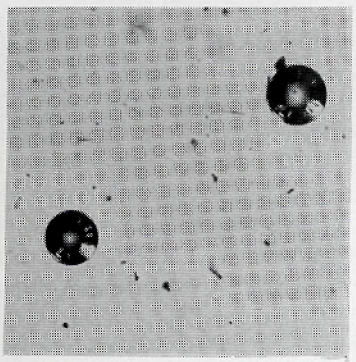

Figure 8 Air bubbles; with top lighting 250X

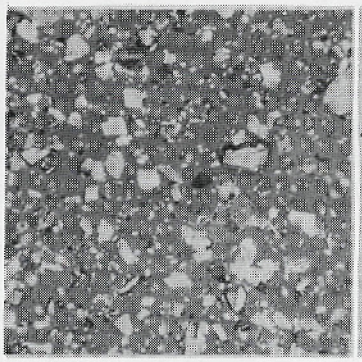

Figure 14 Calcite 250X

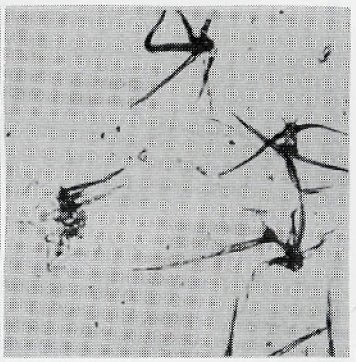

Figure 15 Trichomes 250X

Figure 9 Facial tissue fibers 250X

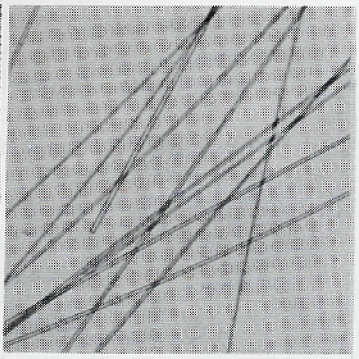

Figure 10 Glass fibers 250X

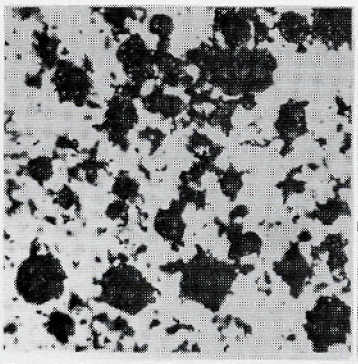

Figure 16 Oil soot; with top lighting 250X

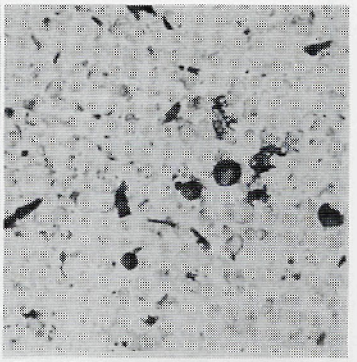

Figure 17 Pulverized coal flyash; top lighting 250X

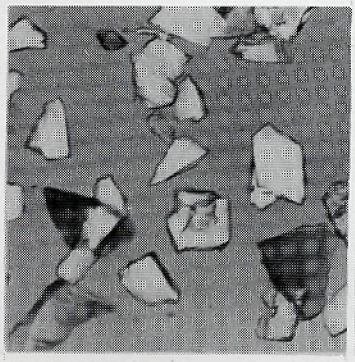

Figure 11 Quartz 250X

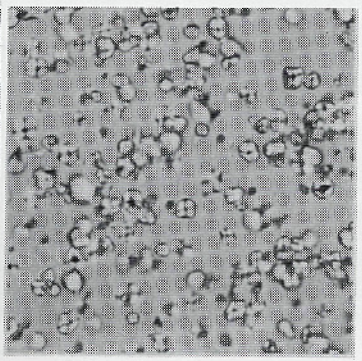

Figure 12 Cornstarch 600X

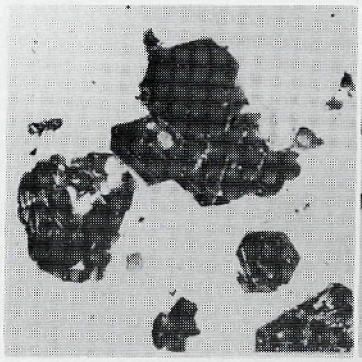

Figure 18 Graphite; with top lighting 250X

Figure 19 Insect parts 250X

Figure 7. *Glass fragments* are transparent, colorless, with sharp edges and corners, equant or flattened but rarely elongated. The fractured surface is rippled or conchoidal. This artifact generally comes from the cover glass or a container and is isotropic, with an index below 1.66. (Some leaded glasses have indices above 1.66.)

Figure 8. *Air bubbles* are perfectly circular in low-viscosity media. They can be distorted by contact with particles or by "trapping" them in a high-viscosity medium. Air bubbles are dark due to bordered total reflection, with a bright center. Shallow air bubbles show only a dark outline with a large bright center. The easiest way to identify them is by pressing on the cover slip, causing them to move or deform.

Figure 9. *Facial tissue fibers* in slide preparations are often from the tissue used to clean the slide and cover glass. Most facial tissues consist of bleached, chemical softwoods. These birefringent, ribbonlike fibers are translucent, colorless and occur individually rather than as bundles. One or two rows of pits, sometimes bordered, can be seen on the longer fibers. Hardwood fibers are usually a minor component of facial tissues. Their presence can be detected by vessel elements which are large, filmy, baggy cells with many rows of pits. All of these elements are birefringent with indices below 1.66.

Figure 10. *Glass fibers* are very smooth, uniform, transparent, colorless, isotropic and generally short. Ends are broken and jagged just like those of macroscopic glass rods. The refractive index is less than 1.66. Glass fiber filters are usual in this contaminant.

Figure 11. *Quartz* is a very common atmospheric contaminant. It resembles glass fragments in shape, surface texture and refractive index, but it is distinguished from glass by its birefringence. Quartz particles occasionally have gaseous and liquid inclusions.

Figure 14. *Calcite* crystals usually show high-order white polarization colors; even five-micron particles show second-order polarization colors. Twinning, which is common, is evidenced by parallel extinction bands that bisect the acute rhomb angle. Because one of calcite's refractive indices is near 1.66, the particles seem to disappear and reappear as the stage is rotated in plane polarized light.

Figure 15. *Trichomes* are colorless transparent hairs from grasses and leaves. Species differ greatly in shape - from single tubular fibers to shield-like plates with radiating fibers. Trichomes show first order (to yellow) polarization colors and have indices below 1.66.

Figure 16. *Oil soot* particles are black, translucent to opaque, hollow spheres or cages. They exhibit a moderate luster and can be broken by pushing on the cover glass. The surface of the oil soot varies from smooth to rough and pitted, due to oxidation and temperature exposure differences.

Figure 17. *Pulverized coal flyash* has three main components: 1) black, opaque, rough to uneven, partially coked coal; 2) partially fused coal minerals with white and red areas in a brown to black matrix; and 3) translucent to transparent and colored to colorless glass spheres.

Figure 18. *Graphite* resembles anthracite coal in its high reflectivity, opacity, and blackness. Graphite, however, trends to be platy, occasionally showing a six-sided particle.

Figure 19. *Insect parts*, when large enough, show an organized structure. Many parts are covered with fine hairs. Body and leg fragments are translucent to transparent and colorless to orange-brown. Almost all of the fragments are partly birefringent, with indices below 1.66.

# Near Contact Mode AFM: Overcoming Surface Fluid Layer In Air And Achieve Ultra-High Resolution

Huddee J. Ho, TopoMetrix Corporation

## Introduction

A major goal of Atomic Force Microscopy (AFM) is to achieve nanometer resolution on surface topography. Vibrating cantilever mode (VCM) is an important configuration of an AFM instrument. It was proposed in the first AFM paper[1].

VCM in ultra-high vacuum (UHV) results in true AFM atomic resolution, which reveals atomic scale surface defects such as a single missing atom in a lattice. However, the VCM operation in air has many difficulties due to the surface contamination on the sample and the AFM tip. The most popular operation modes of the VCM are the non-contact mode[2] and the Tapping mode[3]. Both of these have limited lateral resolution in air.

To optimize the lateral resolution of the VCM in air, we studied the surface contamination layer. With the new understanding of the surface condition in air, we proposed a new VCM operation mode, which is called near-contact mode[4]. In this mode, the tip-sample spacing is minimized and the tip sharpness preserved, and nanometer lateral resolution can be routinely achieved in air.

## The Problems

In ambient air, the tip and sample are usually covered by a layer of surface contamination, which has liquid-like properties. When the tip approaches the sample, there is a moment when the two contamination layers are in contact. At this moment a meniscus forms between the tip and the sample, and a strong capillary attraction is abruptly applied to the AFM tip. The magnitude of the attraction is usually in the order of 100 nano-newtons. This can cause the tip to accelerate toward the sample and result in the tip being captured by the sample surface. In an AFM force-distance curve, this phenomena can be seen as jump-to-contact and adhesion.

When the tip is captured, the cantilever vibration stops and the VCM operation will fail. The other problem associated with this process is the hard physical contact between the tip and the sample when the tip is pulled to the sample. Various experiments have shown that such contact can result in severe tip and sample damage.

The problem of tip damage was acknowledged in the early days of AFM. In non-contact mode (the earliest VCM operation), to prevent tip-sample contact the tip is maintained at several nanometers away from the sample with a vibration amplitude of a few nanometers. Although this can prevent the tip-being-captured problem, the lateral resolution is limited by the relatively large tip sample-spacing.

Later, another method (Tapping mode) was proposed to solve the tip-being-captured problem. This mode vibrates the cantilever at a much larger amplitude, usually 100 nanometers. The tip contacts the sample periodically. Although this can overcome the capillary force that captures the tip, the large amount of energy dissipated at the tip-sample contact point can cause significant tip/sample damage, which also limits the lateral resolution.

In ambient air, both non-contact and periodic-contact have their limitations in achieving nanometer lateral resolution. A new method is needed for optimizing the AFM lateral resolution in air. Since small tip-sample spacing and small tip radius is crucial for obtaining ultra high lateral resolution for AFM, our goal is to find a method that can minimize the tip-sample spacing while maintaining the sharpness of the tip.

## The Analysis

To find a better solution, we should look at the cause of the problem more closely. Figure 1 shows a microscopic model of the AFM tip and sample in ambient air[5]. Usually, the surface contamination layer can be considered as having a structure which consists of two different layers. The first is a molecular layer tightly bonded to the surface of a tip or a sample. The second is a liquid-like layer on top of the molecular layer.

Based on this model, the tip sample relationship can be classified into four different positions (Figure 1). "A" is the true non-contact position and "B" is the contamination contact position. In this case, a meniscus is formed between the tip and the sample, and liquid fills the space between the tip and sample. "C" is the near-contact position. In this case the two tightly-bonded molecular layers come into contact. They serve as a buffer between the tip and sample. This is the optimal imaging position because the tip-sample gap is minimized while the sharpness of the tip is protected by the molecular buffer layer. "D" is the physical contact position. In this case, the molecular layers are destroyed and the interaction among all the atoms in the contact region are as strong as in the bulk. Atom transfer among the tip, sample and contamination layers can occur.

Usually, the large capillary force will result in jump-to-contact, which makes the tip go from position B directly to position D. To bring the tip into the near-contact position, we must first prevent jump-to-contact. The simplest way is to use a cantilever with large spring constant. Experiments have shown that the jump-to-contact does not occur for the cantilevers with the largest spring constants.

In order to practically operate an AFM in near-contact mode, we need a method to indicate that the tip is in the near-contact position. Feedback Fluctuation Analysis (FFA) can be used for that. When an AFM is in feedback, there is a certain amount of fluctuation in the feedback signal. By monitoring the amplitude of the fluctuation versus the feedback set-point, the near-contact position can be located. Figure 2 shows a typical FFA curve. The large feedback fluctuation in the middle of the curve indicates the position B, in which the liquid between the tip and the sample causes the feedback to be unstable. The abrupt drop in the fluctuation amplitude in the tip-approach direction indicates the near-contact position.

To maintain the tip in the near-contact position and maintain its sharpness, we also need to preserve the molecular buffer layer. This requires the vibration amplitude to be small. This is to ensure that the energy dissipated in the tip-sample interaction region is not so large as to damage the molecular buffer layer.

**TABLE 1: Comparison of three VCM operation modes**

| VCM Operation Modes | | Non-contact | Periodic-contact | Near-contact |
|---|---|---|---|---|
| Operation | Amplitude | < 10 nm | > 20 nm | < 10 nm |
| | Feedback Set Point | 50% amplitude reduction, or change of phase | 10 % amplitude reduction | 75 % change in phase signal |
| | Feedback Control PID Parameters | Small | Not sensitive | Large |
| | Spring Constant of Cantilever | Medium to High | Medium to High | High only |
| | Detection Method | Amplitude/Phase | Amplitude/Phase | Prefer Phase |
| Working Mechanism | Relation to the contamination layer | At the top of the layer | In and out of the layer | Staying in the layer, near the bottom |
| | Tip-sample interaction force | Attractive force, negative force gradient | Repulsive force, positive force gradient | Attractive force, positive force gradient |
| Performance | Tip damage | No | Yes | No |
| | Spatial Resolution | Low | High | Very High |
| | Uncertainty in Z | Large | Large | Small |
| | Feedback stability | Not stable | Stable | Stable |

*Continued on Page 14*

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# Near Contact Mode AFM

*Continued from page 12*

### The Solution

To optimize the lateral resolution of an AFM in air, the tip should be in the near-contact position. We call this new operation mode *Near-contact* mode. To run in near-contact mode, a stiff cantilever is needed to prevent jump-to-contact. A cantilever with a spring constant of k = 50 N/m is suitable for most samples. To preserve the molecular buffer layer, a small cantilever vibration amplitude should be used. We only need a vibration amplitude that is large enough to provide significant signal-to-noise ratio – an amplitude of a few nanometers is usually appropriate. Next, we need to bring the tip to the near-contact position to minimize the tip-sample spacing. This can be assured by monitoring the FFA curve. In a normal operation, a 75% reduction of the free vibration amplitude is usually a good set point for the near-contact mode. Finally, let us not forget to use a sharp tip for high lateral resolution AFM imaging.

Figure 3 shows the typical images of three different VCM operation modes on the same sample. The sample used was a polished alumina-silicate glass sample. This is a very good sample for testing lateral resolution. It has two kinds of surface features: the larger "cigar" sharp features on the order of 100 nm, and the smaller round features on the order of a nanometer. Both the images and line profiles show that the near-contact mode has the best lateral resolution. In the non-contact image, the ripples are probably due to the instability of the surface contamination. In the periodic-contact image, the loss of lateral resolution is due to the tip damage by the periodic contact.

Table 1 lists the detailed comparisons of the three VCM modes. With a sharp tip (tip radius <5 nm), the difference between the VCM operation modes can be clearly shown for images of 1 µm or smaller. Since the largest number of pixels in an AFM image is usually 1024 x 1024, for image sizes of 10 µm or larger, the pixel resolution is 10 nm. Therefore, we need smaller image sizes to compare their performance.

### Summary

By analyzing the structure of the surface contamination layer, a new method of VCM operation in ambient air, near-contact mode, is developed. It uses the surface molecular layer as a buffer to minimize the tip-sample spacing and maintain the sharpness of the tip. In the near-contact mode, the feedback is stable and the lateral resolution is optimized. The operation of the near-contact mode can be summarized in a "Four-S Rule": stiff cantilever, small vibration amplitude, short tip-sample spacing, and sharp tip. ■


1 G. Binnig, C. F. Quate, and Ch. Gerber, Phys. Rev. Lett. 56, pp. 930-933 (1986).
2 Y. Martin, C. C. Williams, and H. K. Wickramasinghe, J. Appl. Phys. 61, pp. 4723-4729 (1987).
3 Q. Zhong, D. Innis, K. Kjoller, and V. B. Elings, Surface Sci. Lett. 290, pp. L688-L692 (1993).
4 H. J. Ho, Proceedings of SPIE, Vol. 3512, (1998).
5 H. J. Ho, Ph.D. thesis, Montana State University, Chapter 6 (1997).

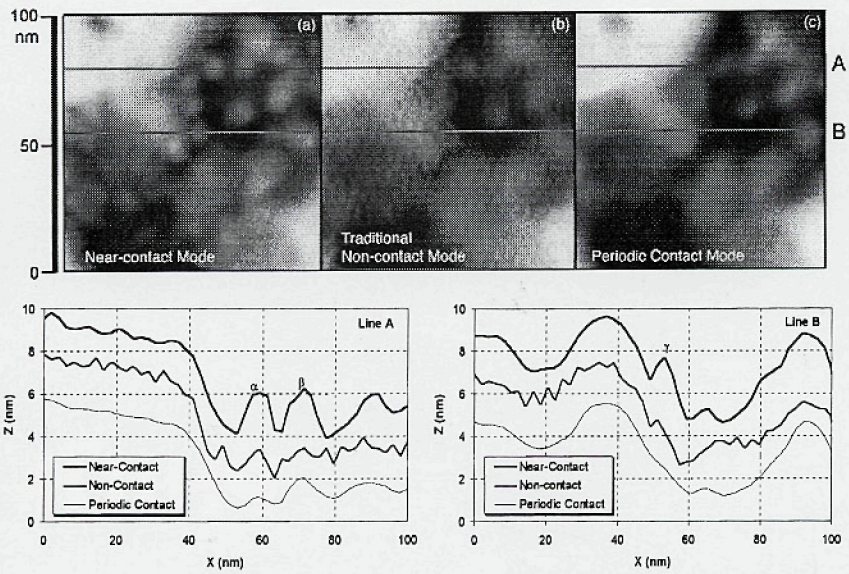




Figure 3: Topographic images and line-profiles of a polished alumina-silicate glass surface with three distinct VCM operation modes. The image size is 100 nm x 100 nm.

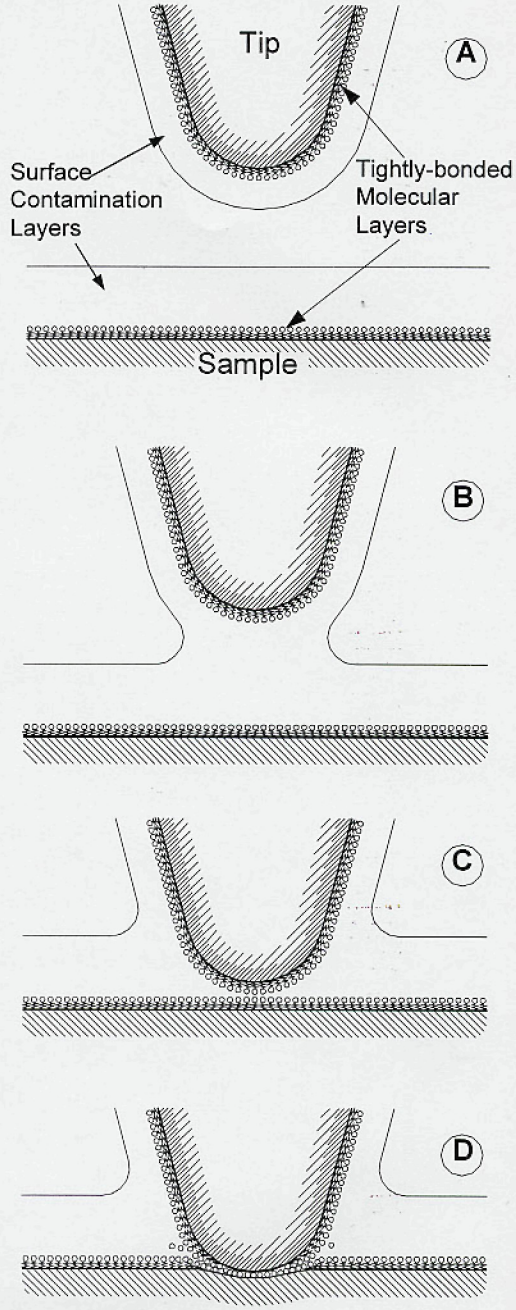



Figure 1. Molecular model of tip-approach process in air.

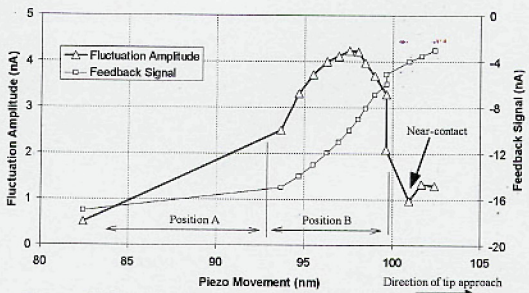
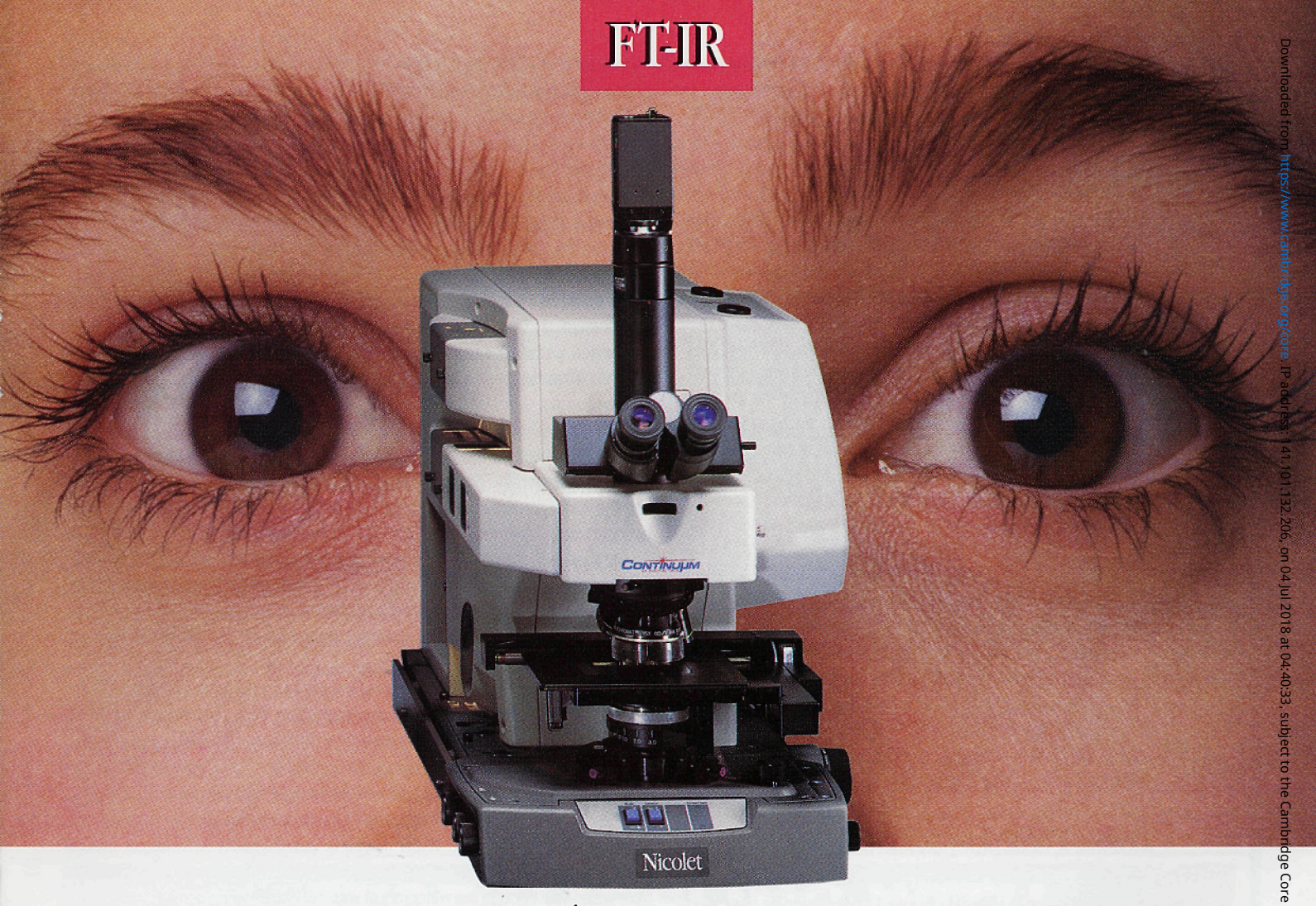



Figure 2: The feedback fluctuation analysis (FFA) shows the transition from true non-contact position to near-contact position. Three AFM tip positions (see Figure 1), A, B, and C (near-contact) can be easily identified from the curve of fluctuation amplitude.

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Zoology and Wildlife Sciences, Auburn University

## INTRODUCTION

Microscopists frequently find that they need to regulate temperature during the course of their experiments. This is especially true for cell biologists working with living cells. Excellent commercial equipment does exist to control the stage temperature of light microscopes, and regulation by such devices is tightly controlled; ± 0.1° C is routinely reported. However, such equipment is often quite expensive and may not provide the investigator with the certainty that the experiment is being performed at exactly the temperature reported by the readout of the thermoelectric control, since the temperature sensor is often at a distance from the actual site of observation. The temperature also varies considerably from the stage heater/cooler to the critical, observed portion of the slide. Since objective, stage and condenser can act as very effective temperature sinks, especially when the slide is fully oiled, the investigator must depend upon a long equilibration time for an accurate temperature readout. Described herein is an inexpensive and easily constructed thermoelectric module that provides very accurate current temperature readout in the Immediate vicinity of the experiment, just outside of the region of illumination and nearly in direct contact with the biological specimen. Although some drift can be expected from the design as described, the design can be easily modified to provide a negative-feedback power supply to regulate the local microscale temperature. A typical current/temperature curve is presented. The device described has been used to study the kinetics of dynein/microtubule interactions using a gilding microtubule assay after the method of Moss *et al.*, (1992).

## MATERIALS, CONSTRUCTION AND CALIBRATION

Overall Construction:

The assembled system is shown in Figure 1. The design as described, including the Lambda power supply, costs considerably less than $500 but will, of course, vary with local machine-shop expenses and materials cost. The assembly can be separated into three distinct sections: a thermally-conductive aluminum slide block (Figure 2), a plastic insulating block holder (Figure 3), and a removable Sylgard™ film that contains the thermistor and provides a fluid space for the biological experiment (Figure 4).

The Metal Slide Insert:

The thermally-conductive slide component is machined of aluminum, and should be anodized to protect it from corrosion caused by biological salt solutions (Figure. 2). Alternatively, and from the viewpoint of toughness as well as corrosion-resistance, the slide can be machined from stainless steel. It is very important to keep the open viewspace in the slide (a 3 mm wide slot at the end of the aluminum or stainless insert) as small as possible in order to maintain the best temperature control.

In the design of the optical and biological chamber, it was important to try to maintain the normal optics and light-path length as close as possible, since the original work was performed at full NA (numerical aperture) under demanding high resolution conditions. The thin end of the thermally-conductive insert was therefore built so that #1 coverslips (22 mm square by approximately 0.1 mm thick) could be fitted to either side of the 0.8 mm-thick section, secured snugly with a tiny dab of vacuum grease, and filled with water to simulate a normal-thickness slide. This satisfys the optical needs of the objective lens. The region of interest in our case could be very small since we were examining microtubule movement, and so a small coverslip chip was further attached to the concave surface of the aluminum slide, again with vacuum grease. The objective viewed the center-most region of the slot, with very little lateral (x-y) displacement possible.

The aluminum block was drilled and tapped to accommodate an acrylic strap that secured the Pettier device. Pettier devices transfer heat across their two flat surfaces dependent upon the polarity of the applied current; one side becomes cold while the other becomes warm. Therefore, although the Peltier device must be tightly attached, a heat-conducting strap cannot be used because heat is carried to or from the slide in a small waterjacket mounted to the reverse side of the Peltier device. A thermally-conductive strap would transfer heat back to, or away from, the aluminum plate and would counter the effects of the thermoelectric module.

The Peltier thermoelectric module (we used a Cambion model 806-1006-01 with an integral water jacket, Cambridge Thermionic Corp., Cambridge, MA) was mounted tightly to the surface, with heat-sink compound (GC Electronics, Southern Electronics, Opelika, AL) to intimately connect the Peltier unit and the aluminum slide. Deionized water was used as the working fluid; the water line being a very flexible thin-walled vinyl tubing that ran from a nearby sink, through the water jacket, back to the sink. Water flowed at approximately 100 mL per minute through the system and did not affect the operation of the microscope. Vibration from water flow through the water jacket was not observed.

The Peltier thermoelectric module was powered by a stand-alone DC regulated low voltage, high current power supply, (model 300, Lambda Electronics, Westborough, MA). A Nobotron unit would also work very well. It could alternatively be powered by an automobile battery through a variable resistor (a "Variac") as are found in many laboratory settings, particularly for electrophysiological applications. The power supply must be able to deliver as much as 9 A at 12 V in order to provide full power to the thermoelectric module.

The Insulating Slide Insert Holder:

The aluminum slide was mounted into a machined polyethylene plastic insulating holder (Figure 3). This isolated the aluminum slide from the stage and allowed the Peltier device to easily displace the slide temperature from the ambient temperature. As a result, the operating temperature of the slide could vary over 0 - 40° C, with temperature changes of 10° C made in less than 2 minutes. Tolerances between the aluminum slide and the plastic insulating holder are not particularly tight, allowing expansion and contraction of the aluminum plate with-

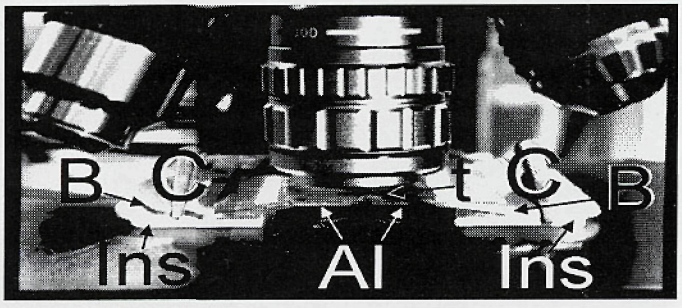


Figure 1: Overview of the thermistor-regulated Peltier-based heating and cooling device. Application is in an upright microscope (Olympus BHS). The flat surface is upward toward the objective, to allow the lenses to freely index in place. Several objectives have been removed for clarity. A high power objective (100X planapochromat) is fitted over the specimen. Code: t, thermistor location; Al, aluminum plate; Ins, plastic insulator plate; C, clip; B, brass thermistor lead extensions.

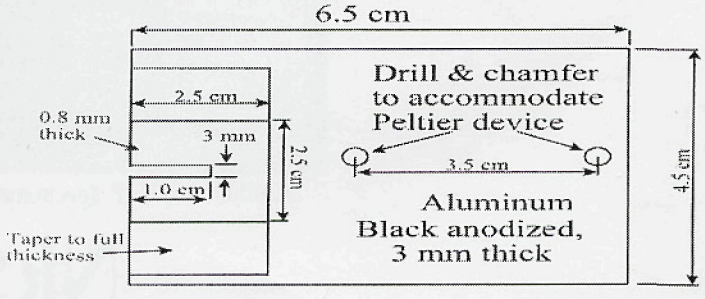


Figure 2: Thermally-conductive aluminum plate. Coverslipped region is in the 2.5 X 2.5 cm square to the left. Peltier device/water jacket (or cooling fins) must be attached to the plate by a nonconductibe strap. In the author's example it was made from acrylic plastic.

out deformation of the plastic assembly, although detectable drift in the aluminum holder must be held to a minimum.

Thermistor Mount:

The thermistor used in this application is an exceptionally tiny and delicate electronic element, and must be protected from damage by embedment in a uniform, tough medium. The black thermistor is encased in a clear glass 350 µm X 500 µm bead with 25 µm Pt-Ir leads. The paradox with the current arrangement is that although rather fragile, it must also be easily removed to allow cleaning of the chamber. Sylgard™, a flexible but tough, polymerizable silicone elastomer (Dow Corning, MI) normally used for a variety of electronic embedment applications, was used to protect the thermistor yet retain ease of removal and remounting. The extremely small, bipolar lead thermistor (Fenwal #GB4IL2; Fenwal Electronics, CT) was embedded in approximately 400 µm thick Sylgard™.The precise overall dimensions of the Sylgard™ film must allow the insertion of the thermistor into the observation chamber end of the aluminum slide near to, but outside of, the illumination path. The thermistor will strongly absorb light and thereby heat up artifactually if placed in the light path. Figure 4 depicts the thermistor/Sylgard™ film assembly.

Brass shimstock (0.001 inch) was used as a tough connecting conductor from the thermistor to the outer edge of the Sylgard™ film. The thermistor leads were carefully soldered in place on the brass, keeping the solder film very hot and thin, and allowing proper cooling characteristics to avoid a cold-soldered joint, while carrying out the process as quickly as possible. Since the thermistor conductors are a 0.001 inch diameter platinum-iridium alloy (and therefore not actually solderable), they were very carefully coiled around the shimstock material prior to attachment, and gently soldered in place. Solder assembly of the thermistor unit must involve great care by the technician since the thermistor is easily damaged by the high heat of the iron. Flat, polished heat sinks must be used between the iron and the thermistor bead to protect the fine wires and the thermistor unit.

A 2 mm-wide channel was cut in the Sylgard™ to allow space for the biological sample, and to allow exchange of fluid via perfusion. The Sylgard™film/thermistor assembly was held in place with a thin film of silicone vacuum grease. The shimstock leads were clamped and stabilized by miniature flat-tip copper-coated spring clips (Radio Shack cat. no. 270-373) mounted to the plastic insulating holder with hot-melt glue. The leads from the clips were made from fine, limp "pillow-speaker" wire to prevent shifting of the slide when mounted on the stage. The leads were then attached to a conventional digital VOM typically found in electronics shops. We used a common model from Radio Shack.

Calibration of the Thermoelectric Module:

Calibration of the thermistor unit was carried out by laying the thermistor/Sylgard™ film assembly in an aluminum boat that floated in the reservoir of a recirculating water bath. The thermistor assembly described here was calibrated at 5 degree intervals over 0 - 40° C. The calibration curve for the described unit is shown in Figure 5.

Operation:

Operation is very simple: The operator attaches coverslips around the thin (0.8 mm) portion of the aluminum assembly, and the aluminum plate is mounted into the plastic insulating holder. The Sylgard™ film is secured with silicone grease into the aluminum plate. The microclips are adjusted to hold the brass shimstock extensions. A third coverslip is mounted in position over the window cut in the Sylgard™ film, allowing the groove to be exposed on either end if the investigator wishes to perfuse the assembly. The entire assembly is mounted onto the stage carrier. Water is applied to the open edge of the 0.8 mm thick region to simulate the full thickness of a glass microscope slide. The author has successfully followed multiple reactivation and microtubule gliding experiments

*Continued on following page*

## A Simple Stage Heater / Cooler for Light Microscopy

*Continued from preceding page*

over a range of 2 to 40° C using this apparatus.

The design given here fits into a microtiter plate holder for the IM 35. Since the observation region is so small (in order to maintain good control over the chamber temperature), the assembly cannot be moved laterally very much. The operator monitors thermistor resistance while adjusting the current output of the power supply. Thermistor resistance is inversely proportional to the temperature (see Figure 5) and the operator can quickly determine the temperature. Although this procedure might at first sound cumbersome, with a little practice the device can be preset to different power outputs corresponding to specific desired temperatures, and quickly and reliably adjusted to a new temperature.

The Peltier/thermistor apparatus can be set up to drive negative-feedback circuitry to automatically regulate temperature, with a concomitant increase in complexity, time-to-completion, and cost. For many applications this is easy to do and desirable; many such control circuits are available through well-known sources (Horowitz and Hill, 1980). Inoue and Spring (1998) list several appropriate references in which various approaches have been presented. In particular, the circuitry of the designs of Chabala *et al.* (1985) and Datyner (1985) describe, in very great and clear detail, the construction of feedback circuitry designed to regulate temperature using a slightly different scheme, by using thermistor-regulated feedback.

### DISCUSSION

Utility of the Design. Considerations and Cautions:

The design described here is intentionally simple, and can be easily fabricated by the investigator. There are many far more sophisticated designs reported in the instrumentation literature (In particular, see the elegant design of Delbridge *et al.*, 1990 in which the fluid is also controlled with regard to level, flow rate, and temperature.) However, many investigators have limited resources (and time and money) and this design has worked very well in a particular application - the reactivation of motility in a gliding microtubule assay. Also, many designs involve the use of a considerable amount of a working fluid for heating or cooling (*e.g.*, Inone *et al.*, 1975) and although they work very well, their complexity is likely to result in more leakage and potential difficulty to the investigator. I describe here a scheme to use water to draw off the heat (if the thermoelectric module is used for cooling); however, depending on the application, the user could choose to use a very efficient finned air heat-sink, such as is used on computer CPU chips. Such fins are available at electronics supply houses such as Allied Electronics (Ft. Worth, TX). Such an approach would entirely eliminate potential vibration from water flow, and would also eliminate the danger of a water leak near expensive components. However, the design described here never produced any significant difficulties.

Another approach, suitable for work under constant temperature, is an air-curtain assembly that heats the entire stage and optical path uniformly. Such a system provides the very best stability, and the very best image resolution, at temperatures that are distinctly different from ambient. It also has been used very effectively for culturing cells directly on the microscope stage, and for following cells for an extended period of time (McKenna and Wang, 1989).

The design described here was originally made for an inverted microscope (Zeiss IM 35) but has been used quite effectively on an upright model (BHS Olympus). Depending upon the resolution requirements of the experiment, the operator has the option to oil or not oil the flat condenser surface of the apparatus and the chamber cover glass. Shifting between upright and inverted designs merely requires the relocation of the coverslipped assay space housing the specimen by inverting it. Oiling of course greatly improves the resolution by increasing system NA, but introduces a much greater heat sink problem, especially

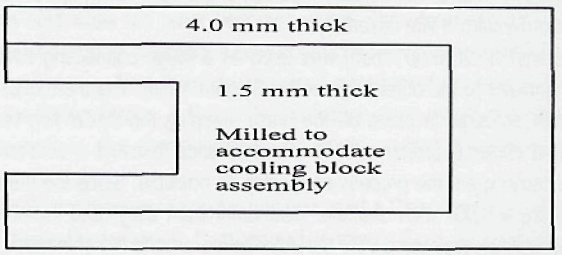


Figure 3: Polyethylene block milled to accommodate the aluminum block of Figure 2. The aluminum block drops into the central area. Dimensions are approximate.

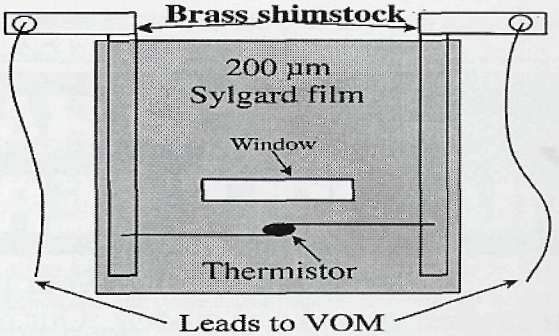


Figure 4: Sylgard™ film embedment of thermistor and brass shimstock assembly. The embedment film was made by building a structure out of plastic coverslips and carefully sealing them to make a flexible mold. Thickness was determined by stacking cover slips and then covering with a glass slide. The assembly was allowed to sit overnight at room temperature to stabilize, then heated for several hours at 60 degrees to assure complete polymerization of the Sylgard™ silicone polymer.

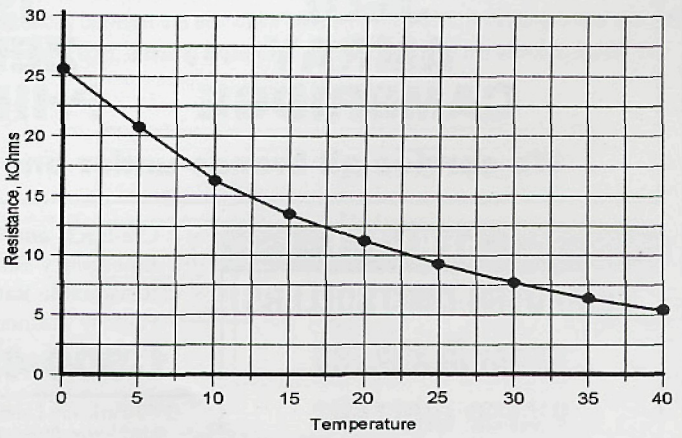


Figure 5: Thermistor response from 0 to 40° C.

if the condenser is also oiled. Furthermore, it is obvious to this author that immersion oils vary in refractive index as the temperature changes; this was, in turn, manifested as changes in focus position and clarity as the temperature was lowered. If the investigator can avoid the use of oil, the temperature control will be much easier with this apparatus. If high numerical aperture is required, substitution of high NA water-immersion lenses, for the more typical oil-immersion objectives, would prevent development of oil/water emulsions that occur in cooled, oiled preparations.

Probably the most difficult aspect regarding day-to-day usage involves condensation of water around the cold objective barrel. Damage to the objective can be alleviated somewhat by sheathing the lens barrel with Parafilm™or a similar wrap. Sufficient insulation to prevent significant condensation requires several layers of tightly-fitting latex glove fingers with a hole cut in the finger tip. This can be difficult and the gloves must be routinely replaced. The opportunity for damage via repeated handlings, the possibility of dropping the lens, etc, make this a less-than-optimal approach.

Long-term exposure of the objective and condenser to fluctuations in temperature can result in loosening of the lens elements, with the unpleasant result that very expensive equipment can be rendered permanently damaged by such experiments. Furthermore, strain-free objectives, such as are used for differential interference and polarization microscopy, that are exposed to temperature fluctuations are likely to develop strain as a function of thermal fluctuations (See Inoue and Spring, 1998 for an in-depth discussion of these dangers) so that their crossed-polars extinction will be greatly attenuated. The investigator needs to balance such effects against the potential payoff of the temperature-control experiment. ■

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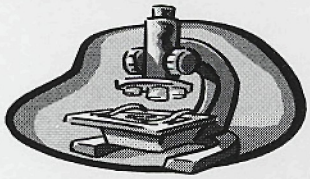

# Home Made Silicone Rubber Embedding Molds

Wolfgang Muss
Institute for Pathological Anatomy
Salzburg General Hospital, Salzburg, Austria

Silicone rubber embedding molds may be inexpensive In North America, but are not necessarily so elsewhere in the world. Further, commercial molds are available only in standard sizes and only with standard sizes and shapes of cavities for specimen embedding. If, however, one needs to embed specimens of nonstandard size or shape, wants more embedding cavities per mold than usually available, or is working where commercial molds are expensive or not easily available, then all is not lost. Embedding molds for specimen blocks can easily be made in the lab from silicone rubber. Further, making embedding blocks allows the use of different silicone rubbers which would be of value with unusual embedding resins, or when embedding must be done in unusual environments (such as extreme cold or heat).

## The Primary Mold

Making embedding molds first requires constructing a "negative mold" for casting the silicone to make the final mold. This primary (first) mold can be constructed of Plexiglas or wood covered with a very smooth surface such as melamine or PVC plastic. "Wood" made of compressed and glued wood chips is not recommended for this first mold. PVC or hard PVC (Acryinitrile-styrole-Acrylnitrile), or resins like Duroplast, Durovit, etc., can also be used for this, but for this note, it is assumed that Plexiglas is used,

The primary mold is sized according to how large the embedding (final) mold is to be, allowing for any size change during curing, so that the mold will fit into any oven used during embedding. This dimension is important!

To construct the primary mold, glue three Plexiglas plates as sides to a base plate. The fourth side will remain free. Add reinforcement to the base and three side plates joints by gluing strips along the outside of the joints.

As the slide-in plate must fit with precision in this three sided box, the three side plates and the baseplate must not have glue on or at the inner sides or edges. Such would interfere with the exact horizontal plane (of the slide-in plate) needed when curing the silicon rubber mass, as well as with the proper and tight fitting of the slide-in-plate with respect to base-plate and side plates when pouring the silicone mass. The surface of the Plexiglass parts should be thoroughly cleaned (fat free) before gluing by wiping with alcohol (*e.g.*, 100% EtOH). Rather than using "regular" glue, use chloroform mixed with Plexiglas "sawdust" or commercially available plexiglass glue to glue the sides. This will dissolve the Plexiglas and form a "weld", rather than a simple glue joint. "Regular" glue might result in excess glue coming out of the joint, and will give messy edges to the final product (including in the embedding cavities, see below). Gluing Is facilitated by using a disposable syringe to apply the glue.

The fourth side is left free, so that a slide-in plate with the forms for the embedding cavities can be placed in the primary mold. After placing the slide-in plate in the mold, this fourth side is held to the mold with clamps and/or with strong self adhesive insulation tape. It is a good idea to wrap the ends with plumber's Teflon tape.

The embedding cavities are made by gluing forms to the slide-in plate. These forms are sized and shaped according to the embedding cavities required. Make the forms about 4% larger than needed to allow for shrinkage during the curing of the silicone mass and/or the embedding resin during polymerization. The forms may be made from any of several different materials, such as metal, Plexiglas, BEEM capsules filled with polymerized resin, and so forth. Be sure to remove the BEEM capsules from the polymerized resin before gluing the resin blanks to the primary mold base-plate. Use as many forms as desired and make as many embedding cavities as needed (see figure 2).

Make identification numbers for the cavities by engraving numbers or letters in normal writing on the tops of the cavity forms. This results in reversed writing on the embedding mold, and in normal writing on the final resin casts. Identification marks can be inscribed in reversed writing on the primary mold or cavity forms so they can be read on the embedding mold.

The primary mold is then assembled: the slide-in plate with cavity forms is slid into the primary mold and the fourth side is then held by clamps or tape.

## Casting the Specimen-Embedding Mold

The embedding mold is cast from silicone rubber. I have found that Elastosil RTV-2 M 4503 with T 35 hardener (Wacker Silicone Co.) at 5% (wt:wt) works well, but other types will work also. Local availability will determine what is used.

The silicone chosen should have these properties:

- ➜ Low to medium viscosity, so it is easily pourable, with a pot-life of at least 15-20 minutes;
- ➜ Moderately "hard", but flexible for easy removal of specimen blocks;
- ➜ Light or pale in color to aid in orienting specimens during embedding (Transparent is also good, then a color background can be used);
- ➜ Should withstand temperatures of 100 to 140°C for rapid curing of resin and "tempering" the molds. Tempering emulates aging of the silicone and increases its stability and life. The silicone and conditions will vary if the specimens are to be embedded at unusual temperatures.

With the Elastosil mentioned above, and the resin polymerized at 65°C, specimens can be embedded, polymerized, and removed by flexing the mold 50 or more times. Polymerizing at 90° to 100° C will shorten the life of the mold.

Follow the manufacturer's specifications for the silicone rubber and hardener/catalyst that you use. Mix in a container (glass or polyethylene) with two to three times the volume of the silicone solution you will be making.

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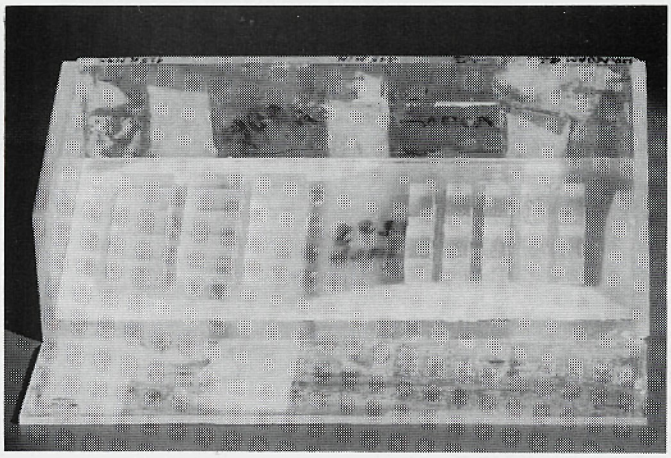

Figure 1A: Primary mold with slide-in plate for making rectangular embedding molds in place. The size of the slide-in plate is 14.8 x 7.3 x0.5 cm.

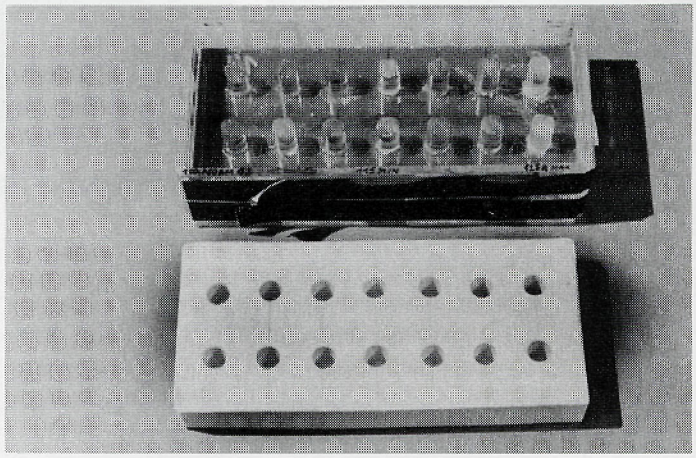

Figure 1B: Assembled primary mold and finished embedding mold for making BEEM-capsule shaped embedding cavities.

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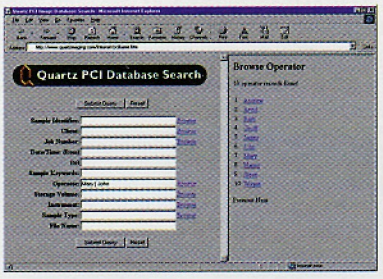


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## Home Made Silicone Rubber Embedding Molds

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Use gloves! The hardener often contains tetra-organo-Zn compounds that can be sensitizing or irritating.

Mix vigorously. The hardener presents an "oily" appearance, and likes to stick to the sides and bottom of the mixing vessel. Be sure that the mixing is complete, and no oiliness is seen.

When mixing is complete, degas the silicone to remove all air bubbles. This is easiest to do in a large vacuum desiccator, empty of desiccant. Line the bottom of the desiccator with paper towels, or spray with a PTFE releasing agent. Don't wipe off spilled silicone, let it cure, and then peel it off. Pumping can be done with a rotary pump, house vacuum, or a hand vacuum pump. The volume of the silicone will increase as it degasses. Admit air before it overflows the container. The bubbles, and so the silicone, will collapse. The trick is to provide a sufficiently large container (2-3 times the volume of mixed silicone rubber). The silicone mass will increase to a point of maximum extension, with lots of bubbles, not like a hydrous solution but more like a very viscous bubble gum mass. After reaching this maximum extension, the silicone mass will collapse back to its pre-evacuation volume. Now it is time to close the valve and admit air (more or less slowly). Don't evacuate any longer since volatile components would be exhausted from the mixture. This could interfere with optimal curing of the mold! If the container is too small to provide the "self collapsing" of the silicone mass, you have to close the valve to stop overflowing the container, then admit air, and then repeat pumping cycles as often as it would be needed to reach the "self collapsing" point of the mass! Proceed as above despite seeing "bubbles" on the surface - they will disappear on pouring.

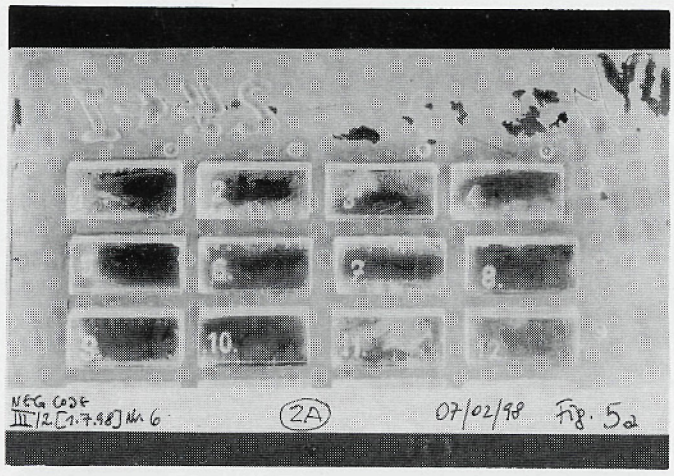


Figure 2A: Detail of slide-in plate for making rectangular embedding cavities, showing specimen block ID numbers engraved in base plate blocks.

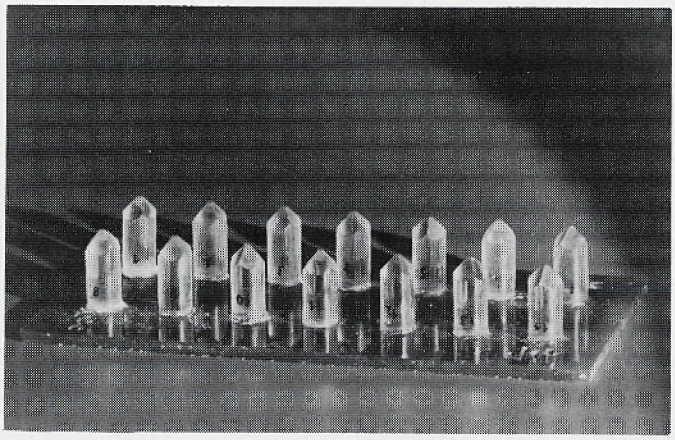

Figure 2B: Slide-in plate used for making BEEM-capsule shaped embedding cavities.

The silicone will be very viscous, but reasonably pourable. A small amount will remain in the container. If needed to fill the mold, this can be carefully scrapped into the mold. Be very careful during pouring not to introduce air bubbles into the silicone. Start pouring slowly, and speed up towards the end of the pour. Start pouring where there are no cavity forms. After the silicone has covered all of the forms, pouring can be speeded up and moved around the mold. Note: be certain that the silicone has completely spread around the bases and edges of the cavity forms before they are covered by more silicone! Otherwise air bubbles will be trapped at these points, with defective embedding cavities.

After pouring, as a product of initial curing, there will likely be air bubbles in the surface of the mold. These will not affect the embedding cavities and can be ignored. They, except for the larger bubbles, will eventually disappear during curing. If desired, these bubbles can be popped with a needle, or "exploded" with a fine jet of compressed air.

To cure the silicone, let the mold stand covered in a perfectly horizontal position in a dry, temperature-controlled environment for 15 to 20 hours. The curing process may be hastened by heating moderately in an oven at 50 to 100°C. This usually is not wise when using plexiglass! After this, soft "fluidy" areas are an indication of poor mixing of silicone and hardener.

Remove the polymerized embedding mold from the primary mold by removing the fourth side of the primary mold, then carefully remove the finished silicone embedding mold from the sides. Remove the slide-in plate and peel off the silicone mold from the molding forms of the slide-in plate. If the slide-in plate is stuck to the base plate, carefully separate the silicone from the sides, and peel the silicone form from the slide-in plate by carefully lifting the mold's edges.

Durability of the embedding mold can be improved if, after condensation, curing is tempered at 120° to 140° C for about 12 to 48 hours - after allowing the polymerized mold to sit for one to two days to finish outgassing curing products.

Note: These conditions, especially the time, may vary depending on the silicone rubber used. Also, polymerization may be accelerated in an oven, its temperature depending on the silicone used.

These silicone molds will last for 20 to 30 embedding and de-embedding cycles, and 50 cycles is easily achieved. The following will lengthen lifespans:

➜ Do not overfill the embedding cavities with resin. The walls dividing the cavities will be destroyed more quickly with overfilling;

➜ Spray the molds after or before use (at least every tenth or fifteenth use) with a thin layer of releasing agent - but don't spray every time, or there will be remnants of the releasing agent left on the specimen blocks. ■

Note: Compound names are for products available in Europe. The same, or closely similar, products are available under different names, from perhaps different companies, elsewhere in the world. In North America, try SylGuard from Dow Corning Chemical Co.

Photographs (Micrographs) by Dr. Arno Laminger, Salzburg, Austria

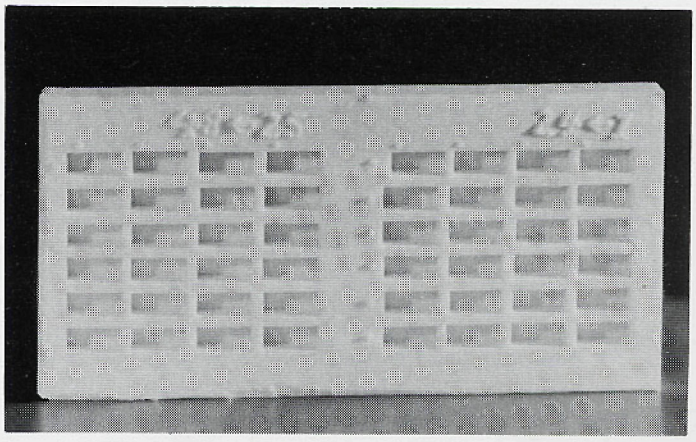

Figure 3: Finished specimen embedding molds showing two possible cavity numbering schemes

**Scanning Microscopy Supplement 10,1996** **ISSN: 0892-953X / ISBN: 0-93128849-5**

## The Science of Biological Specimen Preparation for Microscopy

Edited by: Marek Malecki and Godfried M. Roomans

Proceedings of the 14th Pfefierkom Conference, Belleville, IL, August 1995. Hardbound book with 31 peer-reviewed papers; 476 + vi pages.

Some Comments from book reviews: (1)"... This book is well presented and bound and uses high quality paper to ensure good reproduction of illustrations. The Discussion with Reviewers, which is characteristic of all the books in this series and its associates, is most helpful, as is the inclusion of a subject index and the contents of the proceedings of earlier conferences in this series. This book will make a useful addition to any library committed to maintaining up-to-date reference works on the science of preparing biological tissues for microscopy. Robin Cross, South Africa. (2)"... The final paper is a mammoth review (80 pages) on cryoultramicrotomy by Hans Sitte. The book is worth its price for this paper alone. It is targeted at cryo-observation rather than labeling and covers the whole process from freezing to observation, with comprehensive diagrams of the hardware and some of the best cryosection micrographs ever published. ... Every EM lab should have a copy (and indeed the previous volumes in the series). Guy Cox, Australia. (3)"... This book provides good references and up-to-date information of the highest quality concerning the technical, experimental and theoretical aspects of microscopy and its application. This will satisfy the expanding use of microscopy by researchers around the world." Yutaka Shimada, Japan. (4) "... it is also very good value for money. In fact, it is almost worth it just for the 80 page review of Cryoultramicrotomy by Sitte; you could pay as much for a monograph on this subject. I would recommend this book to all microscopists as an extremely useful source book on specimen preparation for biological imaging and cytochemistry. Julian Heath, U.K. (5)"... The reviewed book represents a honourable continuation of a series of previous publications arisen from these conferences. It is a high quality informative manual, covering a large methodological and instrumental area of microscopy Due to its focus on new, exacting approaches in the analysis of biological subjects at structural, ultrastructural and molecular levels, the book can serve a large community of researchers in the field of microscopy." Jan Korb, Czech Rep.

Contents: Preparation of Plasmid DNA in Transfection Complexes for Fluorescence and Electron Spectroscopic Imaging: M. Malecki (1-16); Simultaneous Identification of a Specific Gene Protein Product and Transcript using Combined Imunocytochemistry and In Situ Hybridization with Non-Radioactive Probes: G. V. Childs (17-26); In Situ Hybridization, In Situ Transcription, and In Situ Polymerase Chain Reaction: L.E. De Bault, J. Gu *(27-47);* Preparation of Samples for Polymerase Chain Reaction In Situ: G.J. Nuovo (49-55); Generation of High Efficiency ssDNA Hybridization Probes by Linear Polymerase Chain Reaction (LPCR): G.W. Konat(57-60); Nucleic Acid Detection by In Situ Molecular Immunogold Labeling Procedures: M. Thiry (61-71); Hydration-Scanning Tunneling Microscopy as a Reliable Method for Imaging Biological Specimens and Hydrophilic Insulators: M. Heirn, R. Eschrich, A. Hillebrand, H.F. Knapp, G. Cevc, R. Guckenberger (73-80); Imaging Molecular Structure of Channels and Receptors with an Atomic Force Microscope: R. Lal (81-96); Atomic Force Microscopy of DNA, Nucleoproteins and Cellular Complexes: The Use of Functionalized Substrates: Y.L. Lyubchenko, R.E. Blankenship, A.A. Gall, S.M. Lindsay et al. (97-109); Microscopic Analysis of DNA and DNA-Protein Assembly by Transmission Electron Microscopy, Scanning Tunneling Microscopy and Scanning Force

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## Combined Pressure-Cooker and Microwave Antigen Retrieval

Allan Kennedy
Glasgow Royal Infirmary University

The use of antigen retrieval techniques has been the foundation of the development of immunocytochemistry (ICC). After the pioneering work done on cryostat sections, the use of enzyme digestion made formalin-fixed archival and current material available for study by ICC. However, the relationship between the duration of fixation and the digestion time necessary for optimum results is a major drawback associated with enzyme digestion. The longer the tissue has been in fixative, the longer the required digestion time will be. Even after a standardised fixation time, some antigens require greatly extended digestion times. Whereas 10-15 minutes in trypsin may be a standard protocol after overnight fixation, immunoglobulin deposits in renal glomeruli may require 60-90 minutes.

The introduction of heat induced epitope retrieval (HIER) methods has done much to avoid these problems. Such protocols do not require modification to cope with extended fixation. Methods have been published using microwave ovens, pressure-cookers and autoclaves to provide the heat source, and all can achieve useful results. The critical feature common to all of these methods is the application of heat to sections immersed in solution. The nature of the solution used is of major importance - heavy metal salt solutions, citrate buffer at pH 6, glycine buffer, Tris buffer and EDTA solutions have all been used. The use of heavy metal salts has been discontinued on health grounds, but the others all have applications in ICC.

Most laboratories use solutions between pH 6 and pH 8, but some antigens give better staining if significantly higher, or lower, pH solutions are used. It is thought that these solutions act by chelating or precipitating cellular calcium which may be involved in cross-link formation during fixation. The heating of the sections ruptures cross-links and thus frees the calcium, which is chelated and precipitated.

In our laboratory we now routinely use a plastic pressure-cooker which fits inside, and is heated by, a microwave oven. This, of course, combines the effects of microwave antigen retrieval with those of pressure cooking. The results have been very impressive. The advantage of steaming is the lower risk of uneven heating (hot/cold spots) when using a domestic microwave. Our steamer can hold up to 1.5 litres of buffer (which is enough for two racks of 25 slides).

We preheat the buffer solution 15 minutes at full power for 1 litre (1 rack of slides) or 20 minutes for 1.5 liters (2 racks). This is started while the slides are being dehydrated, etc. At the end of the preheating period, the solution should be just boiling. We then drop the slides in, put on the lid and the pressure-regulating valve and start the microwave again, allowing about 3 minutes for it to reach full pressure (steamer has an indicator valve to show when this happens), then hold at pressure for 5 minutes.

After the time is up, we remove the steamer from the microwave and let it stand until it has lost its pressure (seen by watching the valve), open the lid and let the solution cool on the bench for twenty minutes before removing the slides. It is likely that the 20 minute cooling period is to allow the reconfiguration of protein molecules prior to immunostaining, and most workers stress the need to include this stage. The same protocol is also proving useful as a pretreatment for *in-situ* hybridisation.

This form of antigen retrieval is invaluable for dealing with referred material, when the fixative and fixation protocol is unknown, and is proving of immense value with a wide and increasing range of antibodies. Perhaps more advances in ICC lie in our use of domestic appliances! ■

# Royal Microscopical Society

**Programme of Forthcoming Meetings and Courses**

| | | |
|---|---|---|
| | **1999** | |
| 25/29 January | Electron Microscopy Course | Manchester |
| 3 March | Annual Immunocytochemistry Meeting | London |
| 11 March | Electron Backscatter Diffraction Workshop | |
| March | Quantitative Measurement of Microstructure | NPL Teddington |
| 25/29 March | 6th International Botanical Microscopy Meeting | St Andrews |
| 30 March | Annual Light Microscopy Meeting | London |
| 12/15 April | 3D & Confocal Microscopy Conference | Heidelberg |
| 14 April | Microscopy of Biomaterials III Meeting | QMWC London |
| 19/20 April | UK SPM Group Meeting | Univ of Surrey |
| June | Immunophenotyping Meeting | London |
| 12 July | FEG Electron Microscopy Meeting | Oxford |
| 19/23 July | Light Microscopy Summer School | Leeds |
| 22/23 July | Immunocytochemistry and Lectin Problem Solving Workshop | London |
| 2/3 September | Lectin Workshop | Oxford |
| 6/10 September | Immunocytochemistry Course | Oxford |
| 13/17 September | Flow Cytometry Course | Sheffield |
| October | Leica 150th Anniversary Meeting | London |
| | **2000** | |
| 3/4 April | Microscopy of Composite Materials V | Oxford |
| 11/13 April | MICRO 2000 | London |
| 16/20 April | EM Spring Meeting | Sheffield |
| 3/8 September | International Congress of Histochemistry and Cytochemistry | York |

For further details about RMS activities, please contact:

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## *Help!!!*

*In our effort to continue to improve the quality of this publication, we truly appreciate reader contributions of articles and materials. The only criteria is that they should be of interest to a reasonable number of our readers. And we also appreciate front cover image contributions.*

*. . . Editor*

# 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:*


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*PO Box 620068 eMail: oshel@terracom.net*
*Middleton WI 53562*
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## Protocol for Preparation of Compact Disks for SEM Analysis

This simple method uses differences in the coefficient of thermal expansion for separation of materials of differing densities. CDs are made up of a metallic core (usually aluminum or gold) surrounded by a plastic layer on either side. Other methods include dissolving the plastic with various solvents, or by removing the metal layer by etching techniques. Both may work fine, I have tried neither. This protocol uses liquid nitrogen to cool the sample (CD) to a point where the materials separate, and has proved successful with both gold and aluminum CDs.

1) *Using tongs, immerse the CD in the liquid nitrogen, after 15 to 30* seconds the CD will sound as if it is cracking. After 30 to 60 seconds, remove the CD from the liquid nitrogen.

2) Place the frozen CD on a firm surface and strike it with a hammer (wear safety glasses). The CD will shatter. Alternatively you may wish to slap the frozen CD down against the bench top (results of the two techniques are similar), shearing between the plastic and metal interface. The metal will easily pull away from the surface of the plastic if still in contact.

3) Mount the metallic layer, which contains the information tracks ("pits" and "lands") on an aluminum stub using double stick conductive carbon tape or tabs. Sputter coating is usually not necessary. Examination with the SEM *is fairly routine at this point (5 to 15 kV).*

**Mike Rock, University of Denver**

## Surface Replication

Here's a good trick. I recently wanted to do leaf surface impressions for stomatal counts and was experimenting with cellulose acetate, etc. A physiologist friend, Todd Dawson, comes along and says "have you tried ignition sealer?" Turns out this works really well, and probably would work for any surface.

I sprayed the leaf surface with Krylon (of Columbus Ohio. 43215) ignition sealant (used as a spray waterproofing treatment for electrical wires and engine components), let it set for 5 minutes, then peeled off the surface coating with clear tape.

The replica/tape was then simply stuck to a glass slide and examined by *phase contrast.*

This works well for epidermal cell shape, stomatal shape and distribution, and vein shape. I don't know how well it will work on trichomes and it probably depends on how elaborated they are. A word of caution; it seems that not all ignition sealer sprays are created equal. 'Wire Dryer" brand did not work at all. Other companies that manufacture this stuff include Hydrosol, Kleenflo and Spray-pak.

**C. John Runions, Cornell University**

## A Stain For Spirochete Bacteria

Here Is a method that doesn't require uranyl nitrate, and it is calibrated for a 700W microwave.

Chemicals:

5% Gelatin
0.15% Hydroquinone
0.5% Silver Nitrate
2% Silver Nitrate

Developer:

Caution: carcinogenic and toxic!
Combine in the order given and prepare just before use:

| | |
|---|---|
| 2% Silver Nitrate | 9 mL |
| 5% Gelatin | 22.5 mL |
| 0.15% Hydroquinone | 12 mL |

Procedure (Time required: 4-7 minutes):

1) Deparaffinize slides to distilled water.
2) Prepare the developer.
3) Microwave slides in 0.5% silver nitrate in a plastic Coplin jar for 2 minutes on power level 1 (10% power, or about 70 Watts) with cap loosely applied. Allow slides to remain in warm solution for 1 minute. NOTE: Fill Coplin jar with 0.5% silver nitrate to the top of the slide slot. Do not go beyond because it will discolor the slide label.
4) Remove Coplin jar with slides from the microwave and discard the solution.
5) Pour the developer into the Coplin jar with slides.
6) Microwave the slides in the developer for 1 minute on power level one.
7) Remove the slides from the microwave and allow them to remain in the warm solution for 30 to 90 seconds, periodically checking that the sections turn yellow to grayish brown.
8) Wash the slides quickly and thoroughly in hot tap water.
9) Rinse slides in distilled water.
10) Dehydrate slides through 2 changes each of 95% alcohol and 100% alcohol.
11) Clear and mount.

Results:

Spirochetes black
Background pale yellow to light brown

References:


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**Rod Green, Grant/Riverside Methodist Hospitals, Columbus, Ohio**

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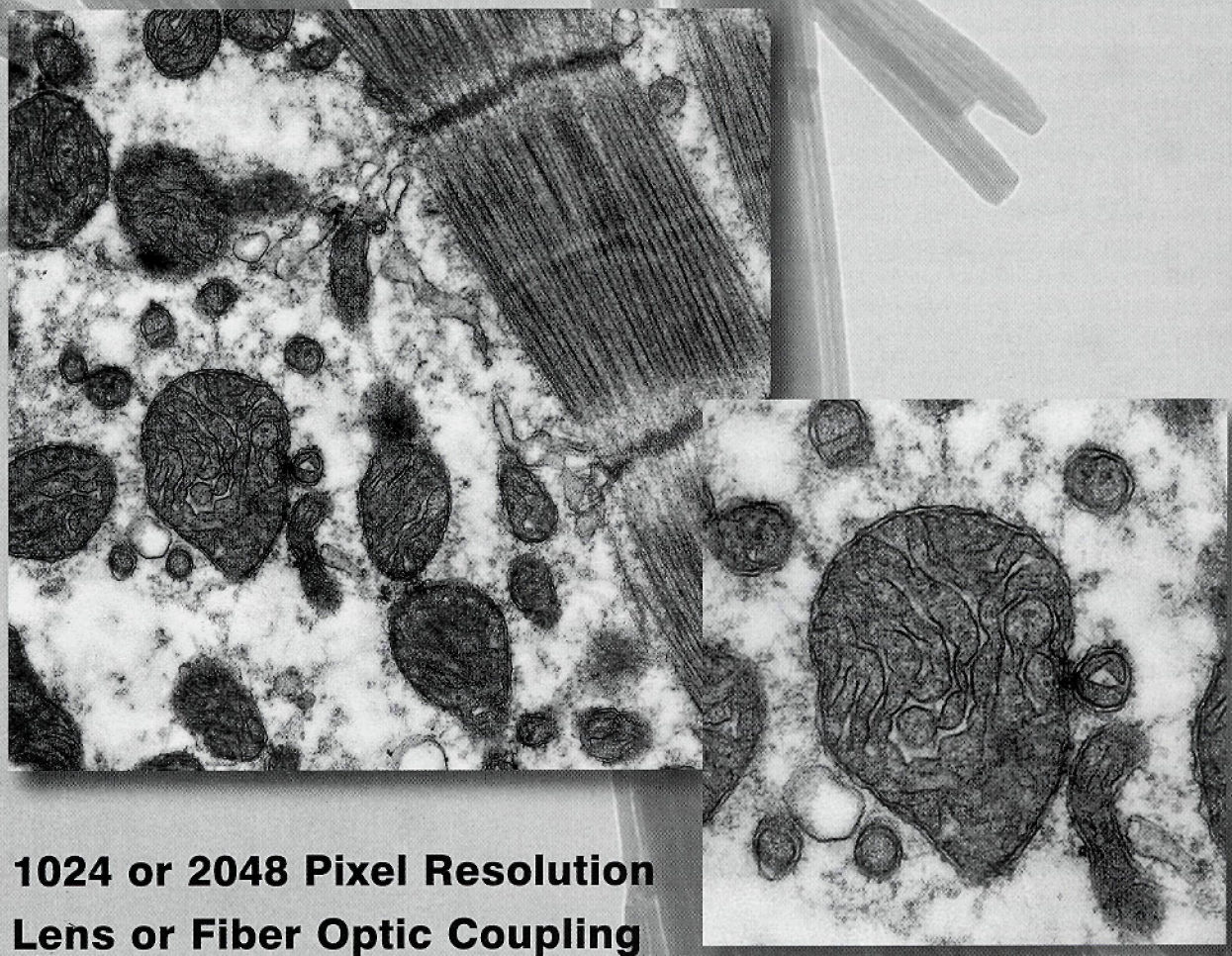

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Circle Reader Inquiry #8

## NEW PRODUCT NEWS

**FEI Company Introduces the DualBeam XL860 Cleanroom-Compatible FIB/SEM Workstation for Rapid-Response Defect Characterization**

FEI Company introduced the all-new DualBeam™ XL860 FIB/SEM Workstation, a completely cleanroom-compatible member of FEI's XL800 family of advanced defect review, analysis, and characterization tools. The new FEB/SEM workstation features robotic sample handling and active environment compensation, combined with extremely high resolution SEM imaging and rapid, precise FIB milling. It is specifically designed for process evaluation, yield management, and failure analysis, supporting design rules in 0.18 microns and beyond. The software automatically handles the complex interrelationships between SEM and FIB operation, allowing the operator to concentrate on analysis of the results

**Reader Inquiry #40**

**Scanning Acoustic Microscopy for under $100K**

Micro Photonics announces the ICAM Scanning Acoustic Microscope, a high-speed full digital and integrated system for rapid, non-destructive inspection of IC packages and similar parts for under $100,000. The ICAM system has been evaluated independently and found to have a substantially lower cost of ownership than competitive instruments. The microscope has the ability to image internal features of a sample showing the presence of manufacturing and processing defects. A unique feature of the ICAM is its high-speed drive that can achieve a reciprocating speed of 12 lines per second on a scan area of 25 x 25 mm. Because of the advanced mechanical and data acquisition design, the ICAM can inspect packages at very high speeds without excessive vibration while generating real time, high resolution images. A scan of 1" square with 250 x 250 pixel resolution takes 20 seconds to perform and images at lower resolution can be completed in even less time.

**Circle Reader Inquiry #41**

**New AFM/STM for 50-500 K Operation**

A new model has been added to the high performance Micro STM UHV Scanning Probe family, offering sample cooling with LHe for both STM and AFM operation using the exclusive OMICRON Needle Sensor technology.

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**Circle Reader Inquiry #42**

**TopoMetrix Introduces Pulsed Force Mode (PFM): An extension of the Atomic Force Microscope to allow measurements of surface properties.**

TopoMetrix Corporation introduced a new imaging mode that extends the capabilities of the Atomic Force Microscope (AFM) beyond simply measuring topography. Pulsed Force Mode (PFM) allows the AFM to measure surface properties such as local stiffness and adhesion with the same high resolution obtained in topographic images. PFM avoids surface damage caused by imaging in contact mode on soft sample surfaces. PFM is sold exclusively by TopoMetrix.

Since its inception, PFM has been successfully applied to a variety of different samples. The PFM control unit allows the user to define a reference from which the adhesion and stiffness values are determined. The topographic image is collected simultaneously by the TopoMetrix system. The PFM-electronics extract the important features of a force-distance cycle. The result is an image of the adhesion, stiffness, and topography of a sample surface all obtained in one quick pass.

The PFM option is manufactured for TopoMetrix by WITec Gmbh in Germany. Additional information on PFM is available on the TopoMetrix web site at www.topometrix.com

**Circle Reader Inquiry #43**

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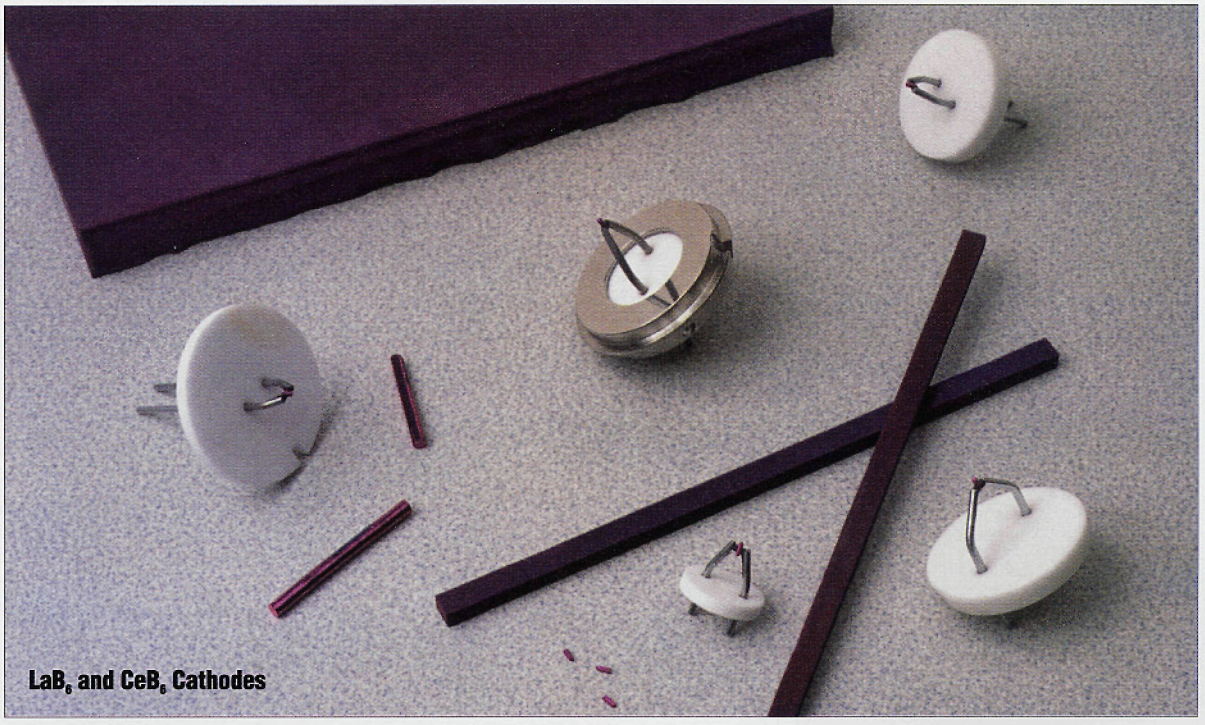

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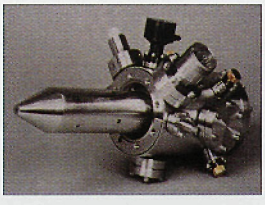

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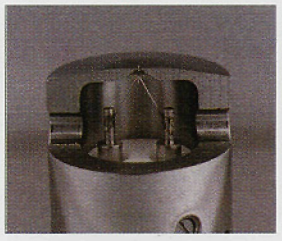

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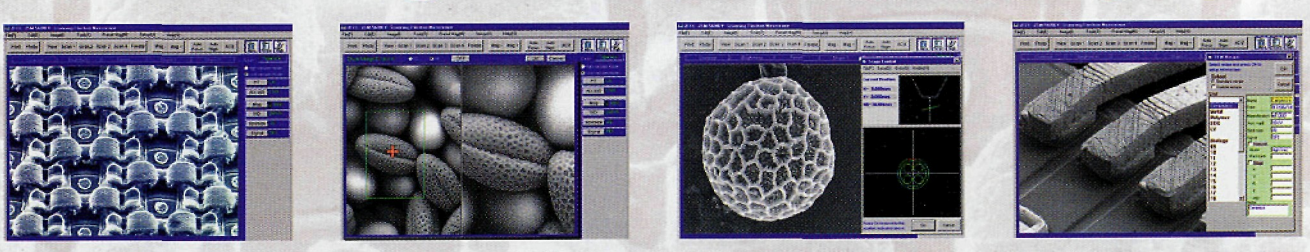

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- ***No Nonsense Transition Between Conventional and Low Vacuum Modes.***
- ***Built-in DTP System Allows Quick and Easy Report Generation and Printing.***
- ***A Special High Sensitivity Detector Allows High Image Quality In All Modes.***

The new JSM-5600 and JSM-5600LV Scanning Electron Microscope was designed from the ground up as a PC SEM. This means that it can be controlled completely and efficiently from only the keyboard and mouse. Keeping in mind the wide variety of preferences for controlling various aspects of an SEM we have also included an optional knobset for multitasking and fine control. You choose.

If you are currently in the market for a high end conventional or low vacuum SEM and need to combine state-of-the-art electronics with the versatility of computer control, we would like to invite you to come to our Applications Facility so that we can prove to you that it really is "As Easy As It Looks".

JEOL USA, Inc., 11 Dearborn Road, Peabody, MA 01960
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