Retention of Calcium and Other Ions for Microanalysis by Freeze-Drying

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Calcium (Ca^{++}) and other diffusible ions cannot be preserved for x-ray microanalysis if specimens have been preserved by fixation using formaldehyde, glutaraldehyde or other fixatives. Atoms, such as Ca, are not diffusible when they are bound to something insoluble, such as bone or hard granules in cells which are sometimes mechanisms for detoxifying[1]. Ions, loosely bound elements and other components such as sugars and amino acids are leached out during chemical fixation and dehydration by solvents[2,3]. This problem was recognized over a hundred years ago by Richard Altmann[4]. An alternative to wet chemical processing is the purely physical method that involves rapidly freezing the specimen and then removing the frozen water by the process of freeze drying, which is the subject of this article.

The rapid freezing of fresh, untreated specimens, known as cryofixation, forms the basis of an extensive literature[5,6,7]. This is because the rate of cooling is critical and much effort has gone into optimizing results. The objective is to achieve the fastest cooling possible. If this is attained, then the water in the specimen can be vitrified, *i.e.*, it solidifies amorphously without forming ice crystals[8,9]. This is necessary to preserve the *in vivo ultrastructure. Slower cooling enables ice crystal nucleation* and growth. Sometimes this can be highly disruptive of ultrastructure. The crystals are composed of pure water and they therefore dehydrate the areas where they grow, displace and rupture membranes and translocate chemicals including the dissolved ions, concentrating them between the crystals. These are therefore concentrated at locations other than where they were originally – artifacts.

The smaller the specimen, the faster the cooling: this applies to all rapid freezing methods. Ideally sized samples would be approximate 0.5 mm cubes or, preferably, smaller. If specimens are larger, then only a narrow surface layer will freeze well, from 5 to 15 µm in depth. This applies particularly to large-area, thin tissue slices. Time-resolved freezing can capture fast cell events such as the moment of synaptic discharge, where a difference of two milliseconds makes a difference[10].

Freezing can be done by plunging specimens into liquid coolants, by impact onto cold metal blocks, by jetting coolant onto them, or by *spraying small specimens in microdroplets into* liquid coolant. Specimens can also be frozen under very high pressure (2.1 kbar), where the physical characteristics of water are changed momentarily so that crystal growth is reduced. Specimens are not plunged directly into liquid nitrogen, because it is already a boiling liquid and forms a layer of gas the instant that a specimen enters the liquid. This acts to insulate the specimen for a short period of time, thus preventing rapid cooling.

Various commercial freezing devices are available from Leica, RMC Inc., Med-Vac Inc., Gatan Inc., Ted Pella Inc., and Balzers-Union. The easiest method to set up on a do-it-yourself basis is probably the plunge freeze method, where a minimal amount of workshop help is necessary[11].

Frozen specimens can be dehydrated by various approaches to freeze-drying, either using commercial equipment such as the Leica AFD or some homemade system[5,6,7]. It is important to keep the temperature low during freeze drying; we use a home-made system which maintains -80° C by circulating liquid nitrogen under thermocouple control. The important aspect is to keep the specimen below the temperature of "freeze drying collapse". This is often held to be -60° C but is probably much higher – the secondary growth of ice crystals by migratory recrystallization of water molecules in the frozen state should be prevented at this temperature. We have observed freeze drying in the cryoSEM up to remarkably high subzero temperatures before the specimen undergoes structural distortion. There are several important temperatures in EM cryo-methodology: amorphously frozen water in vitrified specimens converts to cubic ice at approximately -130° C, and cubic ice converts to the hexagonal form at approximately -80° C. These transitions are not fixed points but are thermodynamic and depend on the rate of rewarming. A thorough investigation of aspects of re-warming showed new translational mobility of water molecules and exotherms occurring at temperatures as high as -6° C, depending on the gel under study[12]. These probably relate to the real "freeze dry collapse" phenomenon.

After the specimens are dried and brought to some higher temperature, often room temperature, they are infiltrated with resin. We generally use Spurr's mixture because of the lower viscosity. Infiltration is routinely done in the chamber of an old vacuum unit. The vacuum normally used is 10^{-4} torr produced by the rotary pump. This is done gently because the resin can de-gas and produce a lot of "froth"!

We cut thick sections 1, 1.5 or 2 microns thick on dry glass knives. 0.5 micron sections are cut but they are more difficult to obtain free of wrinkles. Usually they need to be cut at minimum speed on the ultratome, lifting the first-cut edge with fine tweezers and gently stretching the section during cutting. The thicker sec-

Continued on page 16

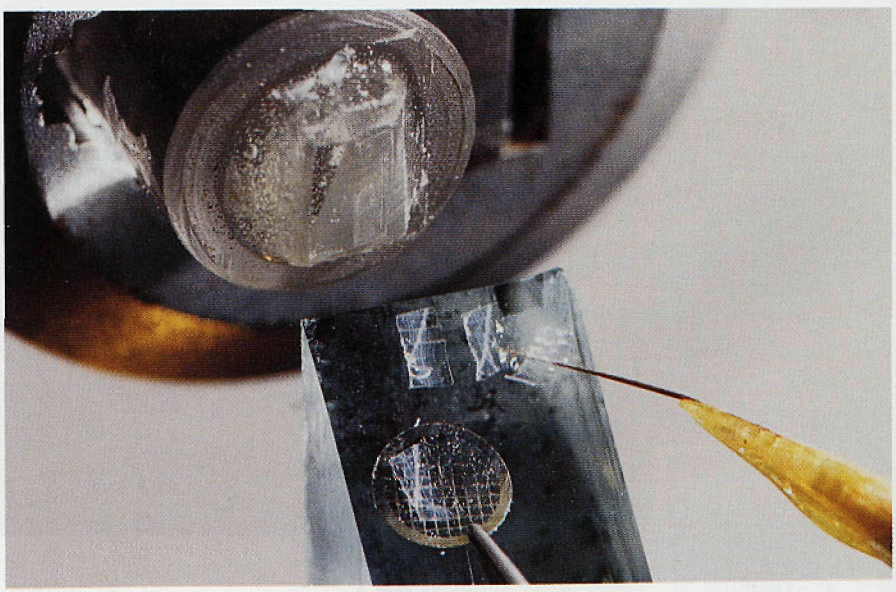

Figure 1. Thick sectioning: 2 µm-thick sections laying just behind the cutting edge of the glass knife. The sections contain a millipore filter bearing algal unicells, *Cephalodiscus pelagicus*. One section is already placed on the formvar-filmed grid and another section is seen on the eyelash tool, being moved towards the grid. The sections would then be pressed flat, using a polished nylon rod.

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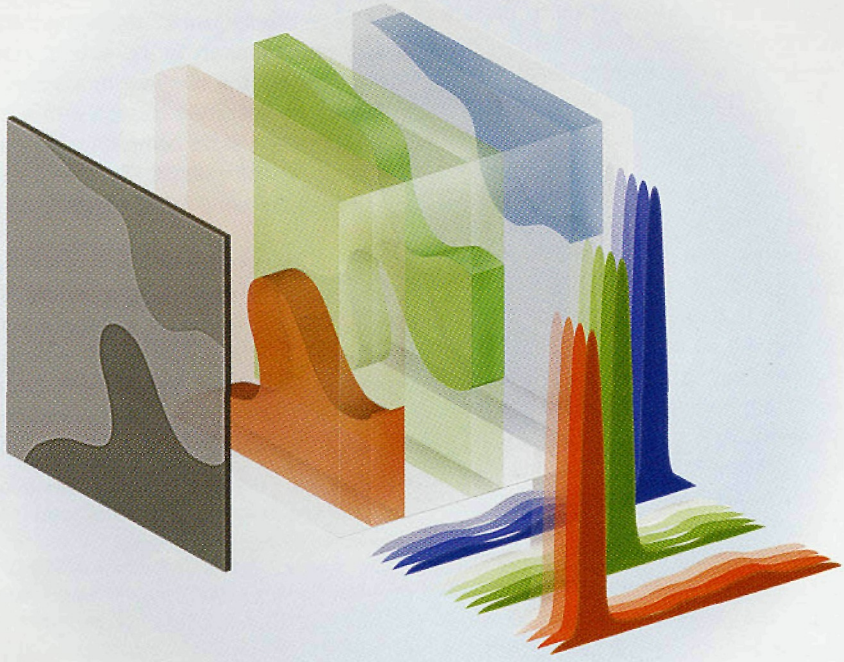

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Retention of Calcium and Other Ions for Microanalysis by Freeze-Drying

Continued from page 14

tions are normally cut rapidly, by hand cycling the ultratome. The 1 to 2 micron-thick sections were cut originally to include hard phosphate granules in metal detoxification studies[1].

Grids are normally formvar-coated aluminium because the grids are likely to fluoresce and contribute to the x-ray spectrum and aluminium was not an element of concern.

The grids are held in reverse-action or clamped forceps (with a small O-ring or a short length of rubber tubing) and held on a lump of Plasticine so that the grid is just behind the knife edge. The sections are manipulated onto the grids with an eyelash (Figure 1). They are pressed down with a polished metal rod although one technician used to deftly "patch-weld" them to the film by pressing them on with the tip of the eyelash. Sometimes, if sections stick to the rod, we sandwich them between two grids, press and separate, although this technique is normally reserved for hydrated cryosections.

Thin sections can be cut from the blocks by normal methods and contrasted with osmium vapour and stained further with uranyl and lead stains for better ultrastructural information. It is probable that conventional ultrathin sectioning with a water bath will leach elements from the surface layer of the block if it gets wet. This would spoil any subsequent sections taken for x-ray microanalysis. Trimming deeper into the block can overcome this problem. The sections prepared in this manner are not used or x-ray microanalysis for three reasons: (1) the diffusible elements will have been lost by exposure to water, (2) the heavy metals will add several predominant peaks to the x-ray spectrum, as is clear in Figure 2, (3) ultrathin sections by definition have little mass and therefore give low x-ray count rates.

There are microprobe considerations to be borne in mind regarding the sections. We chose thick (1 to 2 µm) sections mainly for count rate reasons. It was also traditional in this lab, after many years of analysis of 1-2 µm diameter cell granules which were, in effect, mineral particles. When these are cut with glass knives, they tend to tear out of the section unless they are totally included within the section's thickness. This work was done on a JEOL 200 CX STEM with a 200 kV capability, which was used routinely. With the thicker sections, there is more material to generate x-ray signal. However, to see anything of the structure – which it must be said, can be "vague"- the high kV is necessary. This enables visualisation of the specimens and in itself enhances the count rate. Lower kV does not penetrate the section so efficiently and also imparts more energy to it thereby causing beam damage.

There is also the question of element migration during analysis. This is something we have not investigated but it should not be dismissed. We have seen instances of chlorine disappearing from iso-atomic droplets which were used as sensitivity standards. It is a similar phenomenon to the sublimation of lead stain at big beam currents, when the contrast drops and the specimen area adjacent to the beam becomes coated with a fine deposit of re-condensed lead. This used to be common when sections were examined at 40 kV in older instruments. To guard against the phenomenon the specimen may need to be cooled in a cryostage as a form of cryoprotection.

The dry-cut sections are carbon-coated prior to analysis, for conductance or anti-charging in the STEM. Obviously, the objective aperture needs to be removed for the analysis, otherwise the spectrum will include the aperture or holder elements and possibly others from spurious x-ray fluorescence in the specimen area. The images do not compare with conventional images but, as shown in Figure 3, the chemical "goodies" should be present! ■

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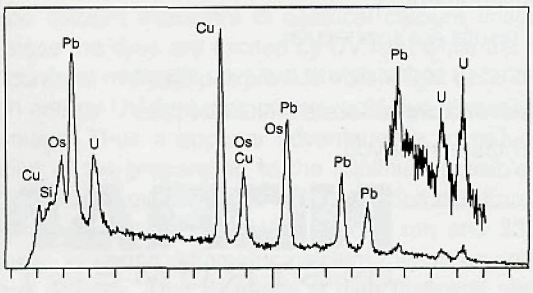

Fig. 2. X-ray spectrum from a specimen prepared by fixation with glutaraldehyde and osmium. The horizontal energy scale is 0 to 20 keV. The vertical scale is 4 k counts, the inset is 250. The ultrathin section was stained with uranyl and lead stains. The peaks from these elements are the only ones apparent in the spectrum, also copper from the grid and specimen rod. The biological elements are either totally lost or overwhelmed by the relative concentration of the heavy metals which have been added to the specimen

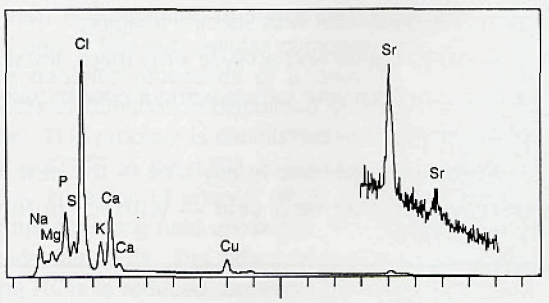

Fig . 3. X-ray spectrum from a freeze-dried specimen of *C. pelagicus*; this marine unicellular alga accumulates calcium to produce a covering of calcareous scales. The energy scale is 0-20 keV. The vertical scale is 16 k counts, the inset is 500. Sodium, magnesium, phosphorus, potassium, calcium and strontium are all of biological interest. The strontium originates from a pulse-chase feeding experiment where the calcium is replaced. The chlorine probably originates from the Spurr's resin mixture. There is some aluminium from the grid, which was mounted in a graphite (low background) holder. The copper probably originates from the microscope.

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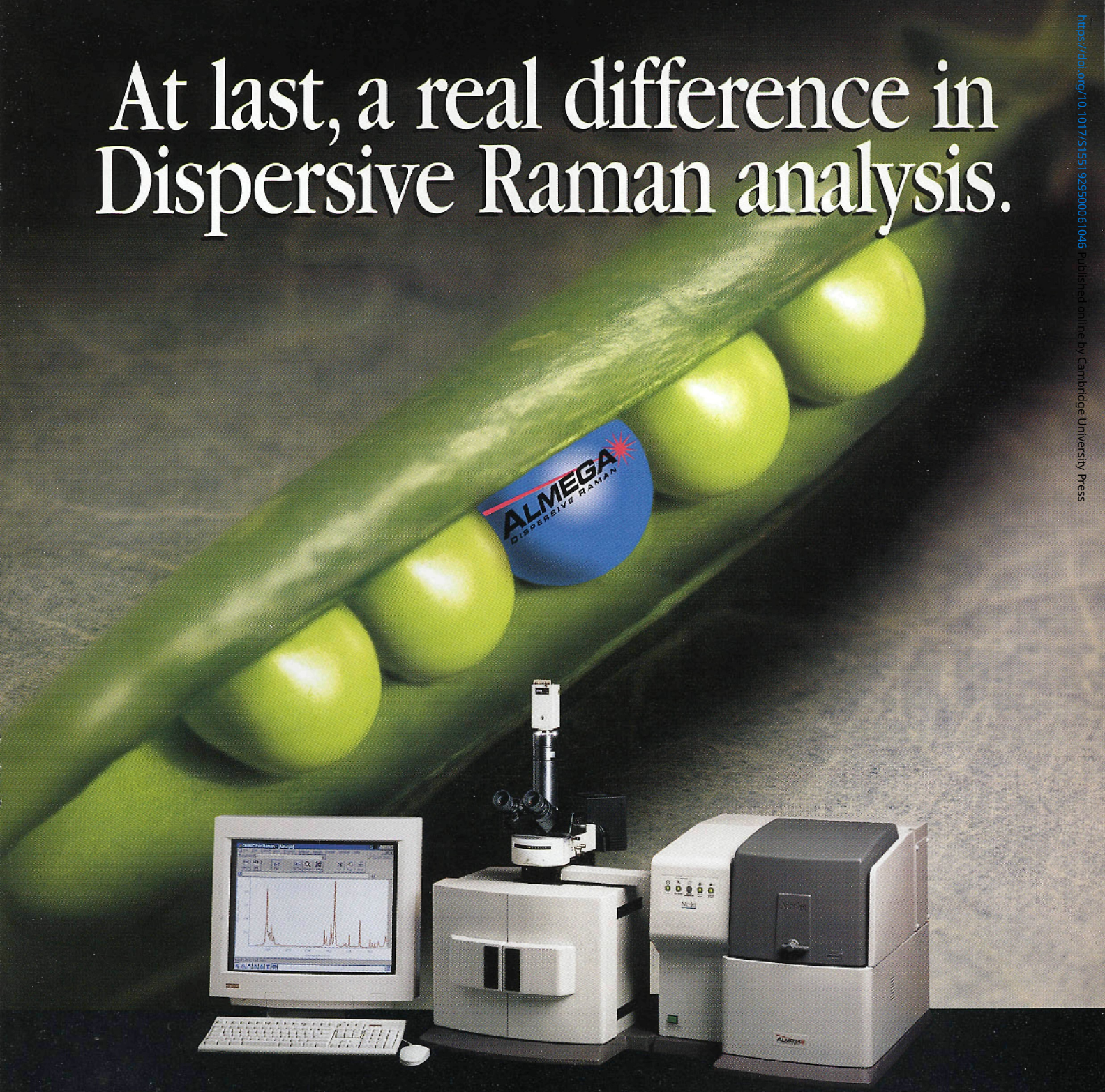

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NEW AND/OR INTERESTING AT PITTCON 2000

As is our custom, and for the hopeful interest of our readers who could not recently attend PITTCON 2000 in New Orleans, we have attempted as follows to summarize what was new and/or interesting in microscopy:

✖ **AABSPEC Instrumentation Corporation** showed their new #S system with the Raman Microscope format. It provides variable temperature sampling in controlled environments from high vacuum to high pressure. A large viewing window is provided so that the entire horizontal surface of the sample can be observed using a short focus microscope objective for optimal signal collection. The #S fits all popular Raman Microscopes. Also shown was the #M Microscope Hot Stage, primarily designed for FTIR microscpes, but the system can also be used with visible microscopes. The #M fits all popular FTIR microscopes. AABSPEC Instrumentation Corporation: (800)783-9380, www.aabspec.com

✖ **Digital Instruments, Veeco Metrology** announced the availability of a fully integrated near-field scanning optical microscope (NSOM). The system is based around the proven technology of the NanoScope® AFM controller and the Zeiss Axiovert 135 inverted optical microscope. High efficiency optics and integrated detectors allow simple, high resolution transmission and fluorescence maps in addition to topography on the nanometer scale. Fiber sensors with micromachined apertures guaranteed to be <100 nm and integrated shear-force sensors are readily available. Also featured were new environmental controls for the MultiMode™ SPM, including a biological heating system for controlling temperatures to 50 degrees C for imaging live cells and other samples in air or liquid; and a polymer heating sytem which covers the melting range for most common polymers and promises to be an invaluable tool for monitoring structural changes on the nanometer scale with varing temperature. Digital Instruments, Veeco Metrology Group: (805)967-1400, www.di.com

✖ **Dolan Jenner Industries**, the leader in fiber optic illumination for light microscopy applications, rolled out it's new line of microscope illuminators. It's new Fiber-Lite MI-150 and MI-30 (150 and 30 watts of halogen light, respectively) attracted considerable interest with their CE/UL approvals, elimination of adapters to connect light guide to light source, low noise and vibration breakthroughs, and extended lamp life. Both models are available with various gooseneck, ringlight and other fiber optic configurations. Dolan Jenner Industries: (978)681-8000, www.dolan-jenner.com

✖ **EDAX Inc** introduced two new systems: Phoenix, the first WindowsNT™ based energy dispersive system, the newest generation of intuitively easy to use, digital pulse processor based, microanalysis system for SEM and TEM, and Eagle µ-Probe, a microfluorescence EDXRF system using glass capillaries. Sapphire energy dispersive detectors, demonstrating the ultimate in light element sensitivity, resolution and reliability. The DX-95, the easiest to use, and most powerful EDXXRF system available today. EDAX Inc: (201)529-4880, www.edax.com

✖ **FEI Company** featured the XL30 ESEM TMP with embedded EDS. This system was used for demonstrations and educational sessions which supported FEI's theme for the show: "ESEM: The LoVac SEM solution for Failure Analysis". FEI's message to failure analysts is that performing imaging and EDS analysis in a low vacuum SEM is different from that in a conventional SEM and users should be aware of these differences. FEI held short sessions that covered the effect of the specimen chamber gas on EDS results, explained how the gas removes the need for conductive coatings, and how LoVac mode secondary detectors work. FEI Company: (503)640-7500, www.feic.com

✖ **IXRF Systems, Inc**. released its new "EDS 2000" software which includes an advanced particle analysis package, material classification and a new style of position tag spectroscopy. IXRF Systems: (281)286-6485, www.ixrfsystems.com

✖ **JEOL USA** announced and distributed literature on its new microscope, the JSM-6700F Near Lens Field Emission Scanning Electron Microscope. The JSM-6700F boasts an NT operating system, a large sample chamber and super high resolution. JEOL also showed its JSM-5000 line of multi-purpose and low vacuum Scanning Electron Microscopes which includes the models JSM-5500, JSM-5600 and JSM-5900. The JSM-5500 which was new this year is a low cost and versatile, windows based PC-SEM which guarantees 4.0 nm resolution at 30 kV. JEOL USA: (978) 536-2270, www.jeol.com

✖ **LEO Electron Microscopes** presented the 1500 series of Field Emission SEMs based on Gemini Electron Optics, the 438 Series Variable Pressure SEM with the new Variable Pressure Secondary Electron Detector, and TEMs featuring in-column imaging Omega filter technology. LEO Electron Microscopes: (800)356-1090, www.leo-em,co.uk

✖ **McCrone Microscopes & Accessories** McCrone Microscopes & Accessories introduced a complete line of Linkam Heating/Freezing stages and Clemex Image Analysis software to compliment an existing line of Olympus microscopes, imaging equipment, and comprehensive lab accessories. Applications for using the modular Linkam thermal stages include forensic melting & freezing point determinations, liquid crystal research and ice cream studies, x-ray technology, Raman spectroscopy, glass analysis, FTIR, and more. Stages include Heating and Freezing, Freeze Drying, Peltier, Shearing, and one for DSC. Major advantages include a wide temperature range from -195° C to 1500° C, easy sample loading/unloading, programming software for automated heating/cooling, and real time video allowing live image capture and analysis. Clemex Image Analysis Software with automated autofocus universal research stage can analyze thousands of particles in seconds at the touch of a button. Applications include particle sizing/counting, thickness determinations, grain sizing, porosity, crystallography, fiber length, and more. Clemex features: mosaic image stitching (builds fields, analyzes them separately, and inputs info into a histogram), intuitive software and auto programming, customized stage patterns, and the multi-layer grab which acquires images at multiple z-levels to form a complete focused image. McCrone Microscopes & Accessories: (800)622-8122, www.mccrone.com

✖ At **MOXTEK, Inc.** the most interest was in their new HT2.2 x-ray window for Si(Li) detectors. The HT2.2 window has better chemical resistance and higher temperature tolerance than their standard ultrathin AP1.3 window. The improved chemical resistance is accomplished with MOXTEK's proprietary DuraCoat™ technology. The DuraCoat also acts as the vacuum seal, and has been proven to be even less permeable to water vapor than standard aluminum coatings. The HT2.2 window can be used at operating temperatures below 70° C, and can be heated in a static mode with no differential pressure to temperatures up to 200° C.

Also introduced was the PF-2500 Silicon PIN diode x-ray detector. This is a large area PIN detector designed for x-ray diffraction applications, but it is also quite well suited to EDS and XRF applications. The resolution is better than 350 eV at 5.9 KeV with 12 µsec shaping time. The active area is 24 mm^2 (mm x 12 mm) for increased solid angle collection. Where required, even better resolution (<240 eV) can be achieved with the PF-700 (7 mm^2) series of detectors. MOXTEK, Inc.: (801)225-0939

✖ **Nicolet Instrument Corporation** reinforced its world wide leadership position with the launch of two new revolutionary products, the Almega™ Dispersive raman spectrometer and the Antaris™ FT-Near Infrared Analyzer. The Almega is fully automated to make dispersive measurements fast, easy and reproducible. Its addition to the Nicolet product line provides customers with a complete Raman solution. The Antaris is a unique combination of analyzer, software, instrument qualification and global applications support. Antaris provides quick and accurate answers that save time and money. Nicolet also unveiled four new Smart Accessories™, expanding the line of intelligent, snap-in-place accessories to 18. The Continuµm IR microscope has been enhanced to include increased video image performance, a new fluorescence illumination option and a new high-sensitivity option for small samples. Nicolet Instrument Corporation: (608)276-6100, www.nicolet.com

✖ **NORAN Instruments** announced its release of the next generation of the popular VANTAGE x-ray microanalysis system. VANTAGE version 2.0 features Spectral Imaging, a new approach for acquisition and visualization of elemental information. Providing a full EDS spectrum at every point in the digital image, this advanced technique produces a virtually unlimited number of x-ray maps and linescans, as well as spectral analysis of any portion of the digital image—even after the sample has been removed from the electron microscope. Dedicated hardware allows data acquisition throughput of over 60,000 counts per second processed and stored, all dead-time corrected. Spectral Imaging features an intuitive single screen approach that makes visualization of its four information quadrants very easy and fast. NORAN Instruments: (608)831-7224, www.noran.com

✖ **NSA/Hitachi** presented two recent additions to the PC-SEM product line: the S-4300 Cold Field Emission SEM and the S-3000 Variable Pressure SEM/Oxford Instruments Particle Analysis System. The S-4300 was equipped with the NORAN Phase ID System, which utilizes the technique of electron backscatter diffraction (EBSD) to capture and analyze electron backscatter Kikuchi patterns (EBKPs). The S-3000/Oxford Instruments system is a completely integrated analysis unit that enable one-button operation for particle identification and classification – up to 500 particles per hour. Like the highly acclaimed S-4700 FE and S-3500N VP PC-SEMs, these systems run through a Windows® operating system, are completely network-ready, and will work with any EDS system using Hitachi's unique "Hi-Mouse" integration. In addition, NSA utilized the PCI Image Management system as the backbone of the network within the booth – as it is used within laboratories and offices worldwide. PCI can manage images from any and all SEMs, TEMs, digital cameras, TWAIN devices, etc. Subsequently, image viewing can be done off-line at any computer terminal connected to a LAN/WAN or an internet browser. NSA/Hitachi: (650)969-1100, www.nissei.com

✖ **Oxford Instruments Analytical Limited** launched additions to their INCA platform to make X-ray microanalysis even easier and more productive. The enhancements to the INCA family include the introduction of INCAEnergy TEM, a new EDX system for TEMs, the addition of INCAWave = the new WDX (wavelength dispersive X-ray spectrometry) product and an enhanced range of INCAEnergy EDX (energy dispersive X-ray spectrometry). INCAEnergy TEM provides TEM users with a range of powerful tools for the generation, analysis and presentation of microanalysis data. Oxford Instruments Analytical Limited: (978) 369-9933, www.oxford-instruments.com

✖ PITTCON 2000 saw the introduction of two new products from **Princeton Gamma-Tech.** The PRISM 2000 line of detectors made its debut with a new large 50 mm^2 active area detector. With 129 eV resolution, the PRISM 50 offers 5 times the count rate of standard 10 mm^2 detectors. Also introduced was MicroImage2 the latest generation of digital imaging and X-ray mapping software for the Avalon family of microanalysis systems. Utilizing a Windows 2000® desktop with the look and feel of Microsoft Office Suite®, the software is familiar and easy to use for all levels of operators, whether for dedicated single user operation, or for multiple occasional users. Princeton Gamma-Tech: (609)924-7310, www.pgt.com

✖ **RJ Lee Instruments LTD** displayed their new TorrSEM variable pressure system, an extremely affordable package including the PERSONAL SEM, multi-quad BSED, variable pressure operating package, and on-line diagnostics. Clicking an icon switches between high vacuum and high pressure operation with no other user adjustments required. Software packages demonstrated included automated materials characterization analysis and an automated inclusion analysis package. The MicroDrive programmable stage for optical microscopy was also shown. RJ Lee Instruments LTD: (724) 744-0100, www.rjleeinst.com

✖ **Spectra-Tech Inc.** introduced the Fluorescence Illumination option for the Continuµm FT-IR microscope. This is the first combination of the fluorescence illumination technique, a research light microscopy method, with FT-infrared-microscopy. The design allows easy switching between visual/IR and fluorescence illumination modes. Fluorescence illumination is achieved using the all –reflecting Infinity-corrected optics used in the Contininuµm microscope. This design allows the fluorescence illuminator to attach in line with the microscopes trinocular mount. The illuminator features a high-pressure mercury burner with 12 v 100W HAL-halogen bulb. A four position turret holds up to four interchangeable cubes for different excitation wavelengths. The fluorescence illuminator is targeted at light microscopists in the biological, pharmaceutical, forensic, and biomedical laboratories. Spectra-Tech Inc.: (800)843-3847, www.spectra-tech.com

✖ **ThermoMicroscopes** continued its emphasis on quantitative SPM measurements using its Proximal Probe Technology™ with the introduction of several new products. These included the Aurora-2™ near field scanning optical microscope (NSOM), as well as a temperature-controlled stage and environmental chamber for existing SPMs. The Aurora-2 offers sub-diffraction-limit optical resolution (30 nm) and, using ThermoMicroscopes' proprietary, tuning fork-based shear-force-modulation, also provides 1 nm topographic z-axis resolution. Its open-architecture platform allows easy access to signals and ready interface to external detectors. The temperature-controlled stage, designed for use with the Explorer PolymerSystem™, has an operating range of -20°C to 300°C. The environmental enclosure, compatible with both the Explorer™ and CP-Research™ series of SPMs, provides a dry nitrogen sample environment to prevent condensation at sub-ambient sample temperatures. ThermoMicroscopes: (408)744-3034. www.thermomicro.com

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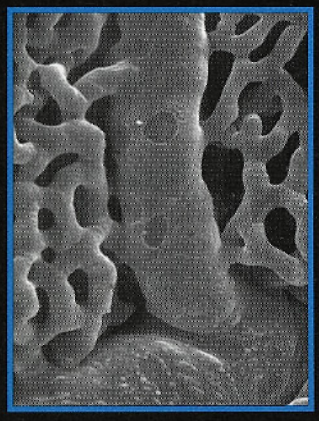

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- ❑ Administrator ❑ Technologist
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3) Your Role in Purchase of Equipment/Services for your Lab/Company: (✓ Check one)
- ❑ Final say ❑ Influence
- ❑ Recommend ❑ No Role

4) Your Area of Science: (✓ check all that apply)
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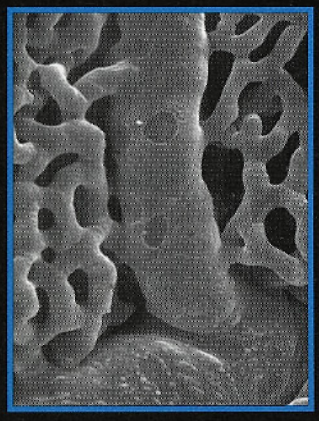

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To register, complete sections 1-9. **Please Type, Print, or Attach your Business Card to section**

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❑ Yes, I will attend the Sunday Reception Ticket included with my Full Registration. (Must be ✓ to receive ticket.)

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