SOCIETY OF
ELECTRON MICROSCOPE
TECHNOLOGY

ALTERNATIVE MICROSCOPIES

2.00 p.m. Wednesday 4th December 1996

EASTMAN DENTAL INSTITUTE
Grays Inn Road
London WC1X 8LD

2.00 **Introduction** - The Chairman

2.05 **FTIR Microspectroscopy - your next step**

Marita Sweeney (Nicolet Instruments Ltd., Warwick)

2.35 **Direct view 3-D optical microscopy**

David Michell (Edge Scientific Instrument Co. Ltd., Milton Keynes)

3.05 **Tea and Demonstration of 3-D Optical Microscopy by Edge**

3.45 **Imaging optical probes for living cell activity using quantitative video & confocal microscopy**

Nicola Parkinson (Dept of Signalling, Babraham, Cambridge)

4.15 **Local probe techniques - beyond the image.**

Phil Williams (Lab of Biophysics and Surface Analysis, Univ of Nottingham)

4.45 **Closing discussion**

5.00 **Annual General Meeting**

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Please reply to the Secretary - Dr Jill Lewis
19 Bellfield Avenue, Harrow, Middx. HA3 6ST
0181 428 4264 (Telephone, Answerphone, Fax)

I hope to be present at the meeting on 4 December 1996

Name .....

Address .....

.....

S.E.M.T. 4 December 1996

ALTERNATIVE MICROSCOPIES

FITR Microspectroscopy - your next step
Marita Sweeney
Nicolet Instruments Ltd, Warwick

The idea of infra-red spectroscopy was first explored in the 1940s, but it took days to collect the spectrum.. Fourier Transform technology was needed.
This is not infra-red microscopy; white light is used for the illumination, then switch to infra-red for analysis. We look at molecular vibrations, which influence the spectra.

FTIR gives speed
can co-add scans
high through-put
computer processing - FT processing
subtraction
library searching

The IRμS^{IM} is a very good white-light microscope. All-reflecting optics are needed, because glass absorbs infra-red rays, with infra-red-transmitting windows. The smallest samples are 5 - 10 μm; the wavelength is typically 2.5 μm - 25 μm.
Because of diffraction, the light intensity is seen in the geometric shadow of the aperture; this gives:
- erroneous low absorption values
- limited spatial resolution
therefore special apertures are needed.

An aperture is used to isolate the area of interest.
Typical specimens include fibres, paint chips, crystals, polymer defects, micro-contaminants.
Molecular information can be obtained. The technique is non-destructive; EM can be done on the same part of the sample.

Transmittance - the most consistent method
largest data base
gives quantitative data

Reflectance - reflaction-absorption
pure specular - Kramers-Konig
diffuse - Kubelka-Munk

The specimen in held flat by windows of salt or diamond.

External reflectance -

Specular - qualitative
quantitative
low reflectance of sample
surface irregularities

Diffuse mixture of specular & diffuse

(another system)

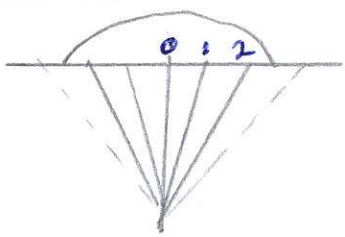
Reflection-absorption can be used for an absorbant layer on a reflective substrate, e.g. printed-circuit board.

Attenuated Total Reflection - IRμS/SIRM scanning IRM

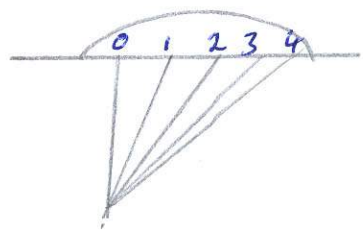
on wheat, brain, polymers, etc. Interfaces rather than interiors.

Direct view 3-D optical microscopy
David Michell
Edge Scientific Instruments Co.Ltd.
Milton Keynes

The Edge R400; high-definition 3-D images by multiple oblique illumination. As light goes through a small aperture, or by the edge of a larger one, it is diffracted, giving zero and several orders of diffraction. To obtain detail in the image, we require at least two orders of diffraction to be collected.



axial illumination



oblique illumination

With oblique illumination, more diffraction orders can be collected, so the resolution in that axis will be increased; with single oblique illuminatkon, increased in the x axis but not in the y axis.
But if there is more than one light source, you can increase the resolution in more than one direction.

The Edge R400 uses four beams, and attains a resolution of 0.2 μm (?), which cannot be attained by conventional microscopes. There is a pyramid mirror in the base of the microscope, to reflect the four light sources.

The results were demonstrated on Tobacco Mosaic Virus crystal, motor end plates in muscle, pollen grain. A fluorescent version of the microscope is also available, and this was demonstrated on bone with two simultaneous fluorescent labels.

Imaging optical probes for living cell activity using quantitative video & confocal microscopy
Nicola Parkinson
Dept. of Signalling, Babraham, Cambridge

She uses an inverted microscope, with dichroic mirror and filter. Fluorescent probes can be introduced into <u>living</u> cells; Fluo-3 and Ca-Green are single-wavelength; Fura-2 and Indo-1 are double-wavelength.

With Ca^{2+} excitation of Fura-2, the peak wavelength moves from 380 to 349.
Similarly with Na^{+} excitation of SBFA, the peak moves from 350 to 330.

The dye is bought as the acetoxymethylester (-AM) or as the free acid (K salt) conjugated with dextran of various molecular weights from 3000 to 70,000. the -AM is lipophilic, and thus can permeate through membranes.

-AM will load all available cells
is non-invasive
simple technique
but there is compartmentalisation of the dye.

Micro-injection - no compartmentalisation of the dye
but only one cell loaded
an invasive technique
technically demanding

A video image takes a composite picture, collecting the fluorescence from everywhere, nucleus as well as cytoplasm.

Osteoclasts are multinucleate, with about 200 nuclei per cell, and have a cytoplasmic "skirt" which contracts to pseudopodia.
With Fura-2-AM, the nuclei fluoresc over 15 seconds, because of the calcium content, then the fluorescence fades; this is by the effect of the GRGDS loading.
The technique enables us to follow calcium waves triggered by Integrin.

Confocal imaging is very useful, e.g. on the Noran Odyssey XL, which hasa resolution of 0.5 μm. Video pictures are needed in order to follow e.g. calcium waves; various time frames can be used.
e.g. passage of wave of calcium along cardio-myocyte.
during oocyte fertilisation, waves of spirals of calcium pass repeatedly over the oocyte.

Local probe techniques - beyond the image

Phil Williams
Lab. of Biophysics & Surface Analysis, Univ. of Nottingham

Scanning Probe Techniques, in particular Scanning Tunnelling microscopy, and Atomic Force microscopy. These can give ultra-high resolution; they measure the forces of inter-action, and can be used under physiological conditions including wet.
Binnig & Rohrer, 1980s.
Scanning Tunnelling
Electrons jump from the tip to the sample, producing a flow of current. This is very sensitive to the distance from the tip to the surface of the sample.

Originally the specimens were placed on a graphite surface, but the pictures thus obtained were completely artefactual !!
The substrate now used is gold on mica. There is still debate as to how the hydrated image is formed.
Catalase crystals are examined in controlled humidity; they require water to be present in order to obtain any contrast formation.

Atomic Force Microscopy
is like a record player. The tip is in contact with the specimen surface.
Atomic size features can be imaged, insulators as well as conductors.
e.g. 40 unit peptide in the β-amyloid of Alzheimer's disease.
The molecular forces of interaction can be measured.
For Streptavidin-Biotin binding, use a Biotin-coated probe, and Streptavidin-coated well surface.

Surface Plasmon Resonance
A laser onto the surface will bounce. The system is sensitive to refractive index. (Bio-Rad)
It is possible to put an Atomic Force Microscope on top of the specimen at the same time as a Surface Plasmon Resonance below. The SPR gives information about thickness.

Scanning Near-Field Optical Microscopy snom
uses a 50 μm aperture in a transparent tip. An optical image is obtained.
There is an evanescent field from the subject on a transparent substrate;
internally-reflected light is involved.
The resolution is 100 nm optically; 2 nm (??) in Atomic Force mode.

ALTERNATIVE MICROSCOPIES - ABSTRACTS

FT-IR Microspectroscopy - your next step ?

Marita Sweeney (Nicolet Instruments Ltd., Warwick)

The combination of light microscopy with infra-red spectroscopy has become a powerful tool for microstructural analysis at the molecular level since the advent of fourier transform instruments. I.r.-spectra of microscopic domains provide information about the molecular chemistry of morphologically defined structures. As a result, FT-IR microspectroscopy can be used as a complementary technique to electron microscopy for many organic based materials. So far, the prime users of the technique have come from a chemical spectroscopy background because of the spectral interpretation skills required, often relying on microscopists for the microsample preparation skills required. However, biologists, materials scientists and geologists also need to relate molecular chemistry with the microstructure of a sample. These remain relatively undeveloped areas of application compared to widespread use in the chemical, polymer and pharmaceutical industries.

The theory and hardware of the technique will be reviewed briefly, with emphasis on the IRμS™ dedicated scanning infrared microspectrophotometer. Mapping molecular structures will be illustrated on a variety of biological tissues and polymers as well as applications as surface probe.

Direct view 3-D optical microscopy

David Michell (Edge Scientific Instrument Co. Ltd., Milton Keynes)

Since its invention in the 17th century the optical microscope has played a key role in scientific research. Following a period of empiricism, the diffraction theory of image formation was first described by Ernst Abbe in the 1870's. This led to the development of the first optical microscope based on known optical theory.

We describe a method of utilising the diffraction theory of image formation to create a high definition 3D optical microscope. Using stereo pair projection, examples of 3D images from the microscope will be demonstrated. We will also introduce the latest development in direct view 3D fluorescence microscopy.

Imaging optical probes for living cell activity using quantitative video & confocal microscopy

Nicola Parkinson (Babraham Inst., Cambridge)

There are many ways now available to visualize and determine quantitative changes in the intracellular physiology of living cells. Data acquisition systems can be attached to fluorescent microscopes to provide on line, live imaging of single cells. I shall discuss two different acquisition systems and the use of selective probes, which provide quality data, but may provide different information. The two systems I shall discuss are video and confocal imaging in relation to viewing changes in intracellular calcium.

There is a wide choice of calcium-sensitive fluorescent dyes available. However, these can be narrowed down to two different types; single and dual wavelength dyes. Single wavelength dyes, with single excitation and single emission wavelengths, have mainly been used with confocal microscopes because they generally tend to be excited at long wavelengths by a laser. Dual wavelength dyes have shorter wavelengths in the u.v. range so need specialised lamps which are more available for video imaging. There are two main methodologies for getting the dyes into the cells. The first method incorporates incubating the cells with the acetylmethoxyester form, which allows the dye to permeate the plasma membrane. The second method involves microinjection of the free acid or dextran-conjugated forms of the dye.

Either method of dye loading can be used for video or confocal imaging. Both video imaging and confocal microscopy provides on-line acquisition of living cells loaded with fluorescent probes. Video imaging captures pictures of the entire field of view as seen down the microscope at a relatively slow rate of acquisition (1 image per sec). This enables the user to see global changes in intracellular free calcium in single cells. We have used video imaging to see changes in intracellular calcium concentration in a wide variety of cells such as smooth muscle, osteoclasts, osteoblasts, cultured fibroblasts and cardiac myocytes. The images we saw from some of these cells lead us to believe that there may be differential changes in calcium between the cytosol and the nucleus. However, as the video image takes a composite picture of the entire cell it was necessasry to move to a confocal microscope. This enables the user to take an optical slice through a single cell to see more precise definitions of changes in intracellular calcium with respect to organelles. It can also acquire data at much faster rates, with the acquisition times reduced from video images (seconds) to a confocal slice (milliseconds), to a single line scan through a cell at microseconds apart. This enables the user to identify basal levels of calcium activity which may remain within a single spatial area of the cell, random calcium events such as calcium sparks, and more organised changes in calcium such as calcium spirals and waves.

Local probe techniques - beyond the image

Phil Williams (Dept Pharmaceutical Sciences, Nottingham)

Even when considered purely as an imaging technique, the scanning probe microscope is a remarkable instrument. The diversity of applications in which SPM has appeared in the literature highlights the considerable range of topographic features, and instrumental problems, which researchers have found during their work. This breadth of application has generated a requirement for new image analysis techniques. But SPM is capable of more than just imaging; indeed the "surface force" functionality of the SPM has, arguably, sparked more interest in the biological sciences than its imaging capability.

Over the past 4 years, SPM has matured into a routine and valuable biophysical tool. This talk will highlight the application of SPM to the study of biological and polymeric systems. Examples will be drawn from self-assembled monolayers of proteins immobilized on gold surfaces, polymer hydrolysis studies using a combination of SPM and surface plasmon resonance, protein adsorption and kinetic and ligand-binding experiments. Data will also be presented showing the ability of the SPM to map ligand-receptor interactions over a surface to nanometre resolution.

In this talk, I will also highlight a new and exciting synergy between SPM force studies and computational molecular simulations. Details of the problems, solutions and advantages of such work will be given.

SEMT Meeting December 4th 1996

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List of Registrants

Paul Ansell	Hitachi Scientific Instruments
Andrea Boyd	Dept Oral Medicine, Guy's Hospital
John Bredl	Royal Veterinary College
Judith Brock	Oxford Instruments
Steve Cham	Leica UK
Terry Cooper	Taab Laboratories, Aldermaston, Berks.
Barbara Cozens	Dept Anatomy, UCL
Heather Davies	EMU, Biol. Sci., Open University, Milton Keynes
Anne Drewe	Microbiology, Charing Cross & Westminster Med School
Barry Dowsett	CAMR, Porton Down, Wilts.
Alan Gray	London Hospital Medical College
Tania Hopcroft	Royal Veterinary College
Chris Jones	EMU, Natural History Museum
Louisa Jones	EMU, Natural History Museum
Mike Kelly	Dept Oral Pathol., London Hospital
Gill Lewis	EMU, Eastman Dental Inst
David McCarthy	EMU, School of Pharmacy
Hilary McPhail	Dept Physiol., St Mary's Hospital Medical College
David Michell	Edge Scientific Instruments Ltd., Milton Keynes
Nicky Mordan	EMU, Eastman Dental Hospital
Nicola Parkinson	Dept Signalling, Babraham Inst., Cambridge
Jenny Plummer	BVS, Royal Veterinary College
Rosemary Suswillo	Royal Veterinary College
Lee Scott	Leica UK
Marita Sweeney	Nicolet Instruments Ltd., Warwick
Martin Turner	Epsom, Surreyl
Phil Williams	Pharmaceutical Sciences, Nottingham University
Naomi Williams	Materials Dept., Open University
Bill Clarke	Agar Scientific
Steve Barnett	St. Mary's