26-2-93

SEMT

Society of Electron Microscope Technology

Affiliated to the Royal Microscopical Society

TO PROBE OR NOT TO PROBE

Friday 26 February 1993

at

Imperial Cancer Research Fund
Lincolns Inn Fields London WC2

PROGRAMME

2.00 **Scanning Tunnelling versus Atomic Force microscopy for biological material**
Sandy McKinnon (Cambridge University)

2.40 **Analysis in the transmission electron microscope**
Pam Champness (Manchester University)

3.20 **Chondrules and igneous rock fragments within ordinary chondrite meteorites**
John Bridges (Natural History Museum)

4.00 Tea

4.20 **Intracellular probing for nucleic acids**
Gary Coulton (Charing Cross Hospital Medical School)

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To the Secretary:

Dr Jill Lewis
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I hope to attend the meeting on 26 February l993

Name.....Telephone .....

Address.....

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Scanning Probe Microscopy Techniques applied to Biological/Molecular Systems

Sean O'Shea
Cambridge University Engineering Dept
Trumpington St. Cambridge

SPM techniques are fast maturing into tried and tested methods of analysis for a variety of systems. However, to date the techniques have been far from conclusive in the area of biological/molecular systems. An overview of the use of these techniques in this area will be given along with some speculation as to future applications. With the problems associated with the techniques in mind, it should be remembered that the instruments are unique in that they lend themselves to investigation in near-native environments for a lot of the systems, and that they provide the possibility for controllable local (atomic/near atomic) experimentation. "Probing" is therefore definitely advised, but only as a complementary technique to other analytical methods.

Chemical Analysis in the TEM

P.E. Champness, Department of Geology, University of Manchester, MANCHESTER, M13 9PL

Modern analytical electron microscopes provide, in one instrument, morphological information with a resolution of less than 2 Å and diffraction information and chemical analysis from areas less than 200 Å in diameter.

The chemical analysis can be obtained by a number of techniques, viz, X-ray spectrometry, electron energy-loss spectrometry (EELS) and Auger-electron spectrometry, (AES). Only a few, highly specialised TEMs have AES and the technique will not be covered here.

Quantitative X-ray analyses can be carried out for all elements for which $Z \geq 11$ with a 'conventional' energy-dispersive X-ray (EDX) detector or $Z \geq 6$ with a 'windowless' or 'ultra-thin window' EDX detector by simple modifications of the procedures used in electron microprobe analysis of bulk specimens. In a 'thin' specimen (i.e. one that is transparent to 100 kV electrons) X-ray absorption and fluorescence can be ignored. There are two ways in which the X-ray intensities may be quantified. In the 'materials' approach the observed ratios of the X-ray intensities I_1/I_2 are converted into ratios of weight-fractions C_1/C_2 by multiplying by a constant $k_{1,2}$

$$C_1/C_2 = k_{1,2}\, I_1/I_2$$

The concentrations can be obtained if an assumption about stoichiometry, e.g. $\Sigma C_n = 1$, can be made.

In the 'biological' approach the concentration of an element is deduced from the ratio of the peak intensity, I_1, to the intensity of the continuum (background), W, over a specified energy range

$$C_1 = k_1\, I_1/W$$

The $k_{1,2}$ and k_1 values are normally determined experimentally from suitable standards. For very soft X-rays, i.e. those from elements $Z < 11$, the 'thin-film criterion' breaks down when the specimen is still very thin and the effects of absorption must be taken into account in the equations.

High-energy electrons lose energy as they travel through the specimen. In the EEL spectrum each element contributes one or more characteristic ionisation edges that sit on an exponentially falling background. These edges can be used to identify and quantify the elements present, although the quantification procedure is less straight-forward than for X-ray spectrometry. EELS is particularly useful for light elements because the ionisation cross-section increases as Z decreases. It also has greater energy resolution and greater sensitivity.

Chondrules and igneous rock fragments within ordinary chondrite meteorites

John Bridges
Department of Mineralogy, Natural History Museum, London SW7 5BD

Ordinary chondrites are the most abundant type of meteorite. They are pieces of rock broken from asteroids and are mainly composed of chondrules and chondrule fragments. Chondrules are rounded droplets of solidified molten rock composed of Fe-Ni metal, sulphide, and the silicate minerals olivine and pyroxene. Their origin is unclear. More rare components of ordinary chondrites have been identified. These are fragments of igneous rocks. Microprobe analyses of silicate minerals in the fragments shows they crystallised from igneous melts with high Mg and low alkali contents. The earth's archean lavas have similar compositional characteristics. This suggests that igneous processes operated on the asteroid parent bodies of the ordinary chondrites at an early stage in their development 4.5Ga.

Intracellular probing for nucleic acids

Gary Coulton

Department of Biochemistry, Charing Cross and Westminster Medical School,
Fulham Palace Road, London W6 8RF.

In situ hybridisation is a method for the localisation of specific nucleic acid molecules in cells or tissues. The basis of the method relies upon remarkably specific chemical bonding between complementary nucleotide bases in DNA and RNA duplexes. Nucleic acids are present in cells as double-stranded DNA in the nucleus and also single-stranded messenger RNA in the nucleus and cytoplasm. Duplexes of either DNA:DNA or DNA:RNA spontaneously form under conditions which depend upon the degree of nucleic acid complimentarity, ionic strength and hybridisation temperature and conditions are specific for each duplex investigated. We, as investigators, can take advantage of this phenomenon by utilising the formation of specific duplexes between native nucleic acid sequences and appropriately labelled complimentary RNA or DNA probes. In the case of *in situ* hybridisation, duplex formation occurs within tissues rather than with purified nucleic acid immobilised on membranes.

The principle strengths of *in situ* hybridisation arise from the ability to discriminate the position of targets in tissues. This is characteristic is best achieved using high resolution methods employing non-radioisotopically labelled probes, where single copy genes can be localised in metaphase spreads with relative ease. The second major strength of the technique comes from the ability to adopt multiple labelling strategies so that two or more targets can be localised simultaneously.

This talk will discuss the application of *in situ* hybridisation to a number of different experimental systems, including cytogenetics, gene expression and viral infection. I will discuss the generation and labelling of gene probes, tissues pre-treatments, hybridisation and visualisation at both light and electron microscopic levels. I will at all stages try to concentrate upon the histochemists rather than the molecular biologists point of view.

Scanning Probe Microscopy Techniques applied to biological/molecular systems

Sean O'Shea

It is possible to image in situ, and to image processes in a millisecond timescale. The tip is only 10A from the specimen surface

The problem is to adhere molecules to the substrate without damaging it; the substrate is very important, and the coating must be well characterised to differentiate it from the sample, e.g. graphite.

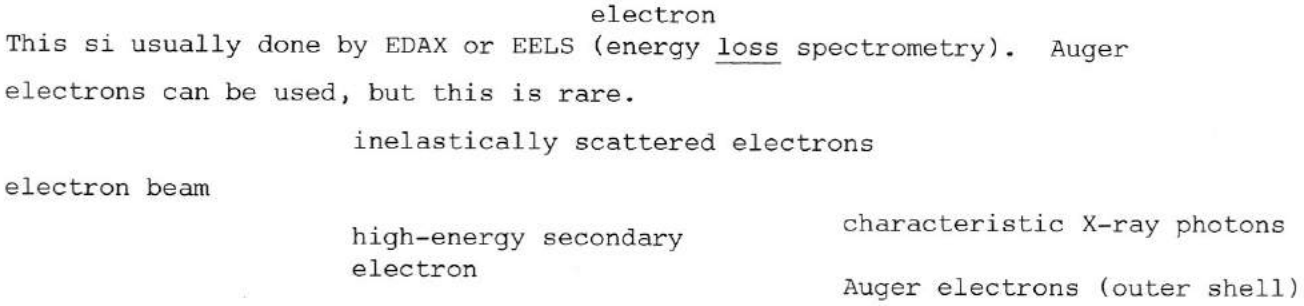
The probe tip moves with the surface, and detects changes in the work function effect below a smooth surface. Resolution is NOT atomic yet, but is in the nanometre range. The contrast mechanism is not understood. So the interpretation of the image is not straightforward.

The Atomic Force Microscope is easier to understand. A laser beam is focuessed onto the back of the lever; a colloid or cone can be fitted on the tip for special purposes. The specimen may get swept aside by the forces as the tip moves over the surface, - pushed or dragged. A range of springs can be used: very stiff for hard surfaces, very soft for liquid or lipid. The image can be distorted because of the tip shape, applied forces, and friction force. Photoemission can also be involved.

The best is a combined STM/AFM microscope.
An STM specimen must be conducting; an AFM can be used on liquids.

Analysis in the TEM

Pam Champness



This si usually done by EDAX or EELS (energy loss spectrometry). Auger electrons can be used, but this is rare.

TEM uses a thin sample, so only a small signal is obtained. An energy-dispersive detector must be moved as close to the specimen as possible.
If a beryllium window is used, elements below sodium cannot be determined.
An ultrathin wondow can now detect nitrogen and oxygen, and a windowless detector for carbon abd boron; it is still not possible to detect lithium at all.

We can rapidly make chemical identification of four kinds of asbestos, and quantify the spectrum.

Electron travelling obliquely to the detector may eb absorbed, especially for the lighter elements.

With EELS, the electrons pass through the specimen and lose energy, so the detector is below the specimen. Spectra can be obtained serially (older, slower method), or in parallel - up to 1000 times more efficient. It is better than EAX for light elements, and gives better speatial resolution. The specimen must be much less than 100 nm thick, but the maximum allowable thickness increases with voltage. It is mroe difficult to quantify than EDAX. The calibration system has a zero-loss peak. We have to model the background, an exponential curve, and subtract this from the total curve; or displace the spectrum by 1 eV, and subtract one from the other.
EELS is better for mapping light elements.
Even with minerals, large dose (= long counting) can remove light elements. High voltage decreases the rate of loss, as does also decreasing the temperature.

Chondrules & igneous rock fragments within ordinary chondrite meteorites

John Bridges

Igneous rocks and chondrites form asteroids, which break to form meteorites. They may be 4560 million years old, dating from the beginning fo the solar system½ On snow or sand, they will stand out from the surrounding rocks because they are black. They are similar to igneous rocks from ancient earth.

Intracellular probing for nucleic acids = in situ hybridisation

Gary Coulton

With biochemical analysis, you use a soup which tells you what is there, but not where it is. In Situ Hybridisation localises specific nucleotide sequences of DNA or RNA, to give you identity, quantitation and position.

Target sequence ⇐⇒ complimentary probe ⇐⇒ reporter molecule

You can use the duplexes DNA-DNA, DNA-RNA, RNA-RNA. The RNA-RNA is the most stable, so that you can use higher temperature and obtain greater specificity.

The probes are: DS DNA, SS DNA, SS RNA (riboprobes), and synthetic dioligonucleotides.

Factors affecting hybrid stabilisation: ionic strength; % guanine/cytosine; formamide (more present = less stable); probe length.

Non-radio-isotopic labels are safe, easier, and cheap; rapid; multiple labelling is possible; you can quantify. The best are biotin-avidin, and digoxigenin-antibody (Boehringer - gives very good back-up).
Photobiotin, from Vector, is useful for initial experiments.

In situ hybridisation can be used on all types of specimen, even those which have already been embedded in wax.
Specimen pre-treatment prevents target loss, preserves the morphology, and allows penetration (proteinase K after fixation). Formalin fixation is better than glutaraldehyde; or keep the concentration of glutaraldehyde low.

A possible schedule for muscle:
cut cryostat sections 8 um
permeabilise with proteinase K in Tris buffer
prehybridise - equilibrate the tissue to buffer without probe:
50% formamide, x2 SSC, x1 Denhart's (polymers), 5% dextran sulphate, 100ug/ml SSS dna, 100ug/ml t RNA
to swamp non-specific sites; equilibrate for 4 hours or more to a plateau, so overnight is OK.
hybridise with 5ng/ml Digoxigen-c RNA at 55'C overnight
wash
RNAse
detect with antibody to digoxigen, conjugated to alkaline phosphatases.

Using multiple probes, 18 human chromosomes can be discriminated separately.

For EM, do pre-embedding labelling; e.g. detect with avidin-ferritin. Or for post-embedding, use Bio-acryl (form Bio-Cell), or LRWhite, or Lowicryl; digestion will leave the RNA sticking up.

Weaknesses of ISH:
sensitivity - 0.1 pico-mol; identity - the mRNA size depends on the person sending the rpobe; need many controls; difficult to quantitate.

Strengths:
spatial resolution; avoids 'diltuion' effect of blots; many more controls are possible, so you learn more; it bridges the gap between gene and protein.

Different targets may require different protocols; you can only tell when you get the signal at the end. At first, use the elast specific conditions.