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SEMT

Society of Electron Microscope Technology

CURRENT TRENDS IN SCANNING MICROSCOPY

Friday, 18 May 1990

at
IMPERIAL CANCER RESEARCH FUND
Lincoln's Inn Fields, London WC2

PROGRAMME

2.00 Chairman's Introduction

2.10 **The use and abuse of the scanning electron microscope**

Mr Steve Chapman (Protrain, Chinnor, Oxford)

2.40 **Preparative techniques for scanning electron microscopy**

Mr Don Claugher (British Museum of Natural History, London)

3.10 Notices

3.15 Tea

3.30 **Applications of 3-D optical microscopy and progress in the 4-D imaging of dynamic cellular events**

Dr Nick White (Dept of Zoology, University of Oxford)

4.00 **Scanning X-ray microscopy**

Dr Chris Buckley (Dept of Physics, Kings' College, London)

4.30 Chairman's Summing up and General Discussion

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To the Secretary:

Dr Jill Lewis, Electron Microscope Unit,
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I hope to attend the meeting at ICRF on 18 May 1990

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CURRENT TRENDS IN

SCANNING MICROSCOPY

ABSTRACTS

THE USE AND ABUSE OF THE SCANNING ELECTRON MICROSCOPE

Mr Steve Chapman (Protrain, Chinnor, Oxford)

When the scanning electron microscope (SEM) arrived on the microscopy scene in the mid-1960's it was rapidly recognised through its ability to provide what looked like 3-D images. This capacity was often used to the full through the observation and subsequent photography of insects and other startling aspects of nature.

Naturally the SEM was compared with its older relation the transmission electron microscope (TEM). The comparison was not often favourable. "The SEM could look ar amost any specimen with little or no preparation." You earned your right to use a TEM by spending months trying to produce a good specimen. "Anyone could operate the SEM, obtain an image and take a photograph," whilst the TEM took years of experience to master. "The big problem with the SEM was its total inability to resolve." In the early days a TEM resolution of 2 Angstrom was being compared with a SEM resolution of 200 Angstrom.

Growing up within this environment the SEM rarely gave of its full potential; very few even looked for it.

In the early 1970's scanning attachments were fitted to the TEM and the resolution barrier was considerably deformed. Developments of this concept, having the SEM specimen within a lens rather than outside, progressed to such a level that by the early 1980's a totally new light was being shed on the formation of an image in the SEM. Having once thought that the image was created simply by elastic and inelastic scatter within the specimen, the new imaging concepts demonstrated how the specimen chamber geometry dramatically altered the image produced.

The old ideas of running an SEM at 15 kEV plus collapsed overnight. Low kV microscopy (<5kV) became the vogue. New data was recognised as it became clear that very rarely in the past had we seen the true **surface** structure. Used in the traditional way an SEM was a **sub-surface** imaging system. Used in the modern style accurate **surface** recognition was being attained. Gone were the simple operating concepts, and destroyed for ever the idea that a TEM is a more sophisticated instrument. Change the kV in a TEM, all you change is the image contrast; change the kV in an SEM, and you may often see a totally different image.

All of these points will be discussed and demonstrated through the use of diagrams and photographs.

The use and abuse of the Scanning Electron Microscope

Steve Chapman

In industry, specimens are routinely examined at x 100,000, and reports back to the factory will affect the production lines.

Originally the SEM image was said to be formed from secondary or back-scattered electrons.. At 10 kV, the beam affects about 0.5 μm of the specimen, in both depth and width; at 30 kV, about 0.3 μm; we therefore get information not only from the surface of the specimen.

Even if we are using a "secondary" signal, most of the electrons forming the image will have been back-scattered. "Electrons can't read". If the image was formed only by attracting electrons into the detector, we would get no shadows in the image.

Backscattered images have high contrast because from atomic number contrast: secondary electron images are soft, with low contrast. The "normal" secondary electron image contains much back-scatter - which does not come from the surface of the specimen.

At 25 kV, the beam will go through a carbon/platinum film on a grid and only the grid will be imaged; at a much lower kV, the film also will be seen. By choosing the kV, we choose what picture we will see. 20 kV is now considered very high: to see the true surface of the specimen, use 5 kV.

(diagram?? shown??)

SE1 from beam entering specimen
2 from back-scattered electrons exiting specimen
3 from lens, generated by back-scattered electrons
4 from aperture, generated by beam - not now a problem.
BS1 line-of-sight back-scattered electrons from specimen
2 electrons bouncing off column components.

The lens tip is a steep cone so as to reduce SE3 and BS3 by reducing the area seen by bse; there is variation between different models of SEM.

Tilt the specimen towards the detector to get more bse from below the surface; tilt away from the detector to get a more truly secondary electron image.

For low kV, the specimen needs to be nearer the lens; low kV backscatter is very useful. Never examine the specimen at only a single kV; Chapman routinely uses 2 and 10 kV, and more if necessary. You can use the kV to "section" the specimen.

It is not necessary to coat specimens for low kV. Chapman routinely looks at the specimens uncoated first, then coated; he doesn't like "grilled" cells (the coating procedure grills them?).

If you have an "old" SEM (more than 10 years old), reduce the anode/cathode distance from 15 to 10 um for use at lower kV, and move the filament forward. But DON'T then use the machine at the higher kV, or you will blow everything.

PREPARATIVE TECHNIQUES FOR SCANNING ELECTRON MICROSCOPY

Mr Don Claugher (Kingston-upon-Thames, Surrey)

This paper presents an overview of techniques that have in the past and may in the future add a further dimension to the uses of the SEM.

When the SEM first became available to the scientific community, its uses were rather restricted to topographical observations, the quality of which varied depending on the experience of the operator. Since that time much has been written about particular techniques, but in this fast-moving world it is difficult to keep up with all that is going on; we tend to stick to particular methods because they suit our purpose. Some of us only look at a limited range of materials and may feel that we have no need for improved methods. This is not always a productive attitude, and when this occurs there is no progress. Progress is based on need and to a certain extent curiosity; these two factors have stimulated a number of innovative techniques to be developed, some of which I will cover, giving the reasons for their development and suggesting areas where they cn be applied.

We tend to think at moments in time that we have reached the ultimate in certain areas of technique but I hope to demonstrate that there is much more to be seen with the SEM than we see at the present time.

For fixed material, critical-point drying is generally better than freeze-drying.

For uncoated material, back-scattered electrons + charge-free anti-contamination system (CFAS) = Wetsem = high-pressure SEM.

Fast atoms will etch even silica; this is used in ion beam and field emission EM.

Chromium oxidises very fast; a chromium replica would lose all detail within two days!!

APPLICATIONS OF 3D OPTICAL MICROSCOPY AND PROGRESS IN THE 4D IMAGING OF DYNAMIC CELLULAR EVENTS

Dr Nick White (Dept of Zoology, Oxford University)

Direct 3D imaging is made possible by a point scanning optical microscope with confocal illumination and detection systems. In this configuration one or more focussed optical points are scanned through a 3D sample in parallel with a similarly aligned and focussed detector. A physical aperture restricts the detector to receive only light returning from this confocal point. This rastered imaging system sweeps out a 2D optical section and when "focussed" through the sample produces a 3D image.

At the SERC - Oxford optical microscopy facility we have been developing 3D confocal techniques, through collaborations involving a variety of biological and bio-materials projects, and have recently begun to combine these with conventional time-lapse methods into 4D microscopic imaging. Digital processing systems such as ThruView, a 3D visualisation program based around the Bio-Rad MRC 500/600 confocal microscope, are being developed to extract useful information from stored image data. Illustrative results arising from these developments will be presented:

The volume and shape of living cells has been studied by confocal imaging of both plant (leaf stomatal cells) and animal (Chinese hamster lung, mouse and human cultured fibroblasts), in fluorescently labelled medium. Non-invasive 4D imaging of, for example, the fibroblast surface morphology during contact-induced spreading or during motility such as in wound healing is achieved by this "negative fluorescence" method.

The distribution of antigens during antibody cross-linking induced capping on the surface of rat thymocytes has been studied by 3D reflection imaging of immunogold labelling. We have looked at several problems with this kind of imaging of living cells including reflection artefacts from cell/medium and glass/medium boundaries and movement of these small non-adhering cells during image collection. 3D reflection imaging of bio-materials, including geological samples and contact lenses has provided non-destructive information previously only available from thin sections.

Cytoskeleton and subcellular organelle distributions are studied routinely by confocal fluorescence imaging and ThruView has played a major role in the development of display techniques for presenting what is often a complex 3D image on a videoscreen or photograph. The ability to animate sequences of images representing views from different directions conveys not only a sense of movement of the 3D image but through motion parallax, a simple extension of stereo display, gives additional depth information not available from single views alone.

The continuing rapid progress in digital equipment design for fast animation and display is enabling us to move closer towards interactive 3D and 4D microscopy of cellular processes.

SCANNING X-RAY MICROSCOPY

Dr Chris Buckley (Dept of Physics, Kings College, London)

X-rays in the energy range 200 - 600 eV (5 - 2 nm) have been used in a scanning X-ray microscope to form images of biological specimens. X-rays in this energy range offer higher resolution than light due to the shorter wavelength. Natural absorption contrast can be used thus eliminating the need for staining. Also, preparations can be imaged wet and whole, as the absorption of the X-rays by water is much less than that for protein. The X-ray microscope is operated at atmospheric pressure, and at room temperature.

In the scanning microscope, the X-ray probe is formed by a modified Fresnel zone plate which acts as an X-ray lens. These zone plates are made at Kings College by electron lithography, and present zone plates are capable of producing spot sizes of about 50 nm. Unlike the scanning electron microscope, the X-ray microscope has a stationary probe and it is the specimen which is scanned. The transmitted X-rays are counted at each position of the X,Y scan. In this way the image is built up pixel by pixel by displaying the recorded counts as a brightness level on a screen.

Scanning X-ray microscopy is a young science, and is presently at the appraisal stage. It is hoped that living cell cultures can be imaged and that the dose required to image living cell cultures will not kill or induce serious morphological changes to the cells. Results presented will include images of fibroblasts and calcium distribution in calcified tendon. Current and future work will be discussed.

λ	10 - 500 A	absorption cantrast
	8 - 40	phase contrast
	0 - 10	fluorescence

Scanning X-ray gives less radiation damage, but needs a high brightness source. At present, cells apparently die within 4 hours, at doses above 10 mRAD.

Elemental mapping is possible.