Electron microscopy comes to life

One important limitation of electron microscopes until now is that the conditions under which the specimen is examined have made it impracticable to view living matter. Results from a technique now under development are showing rapid progress in microscopy of biological materials and are paving the way to observing the dynamics of life at high magnifications. Incidentally, it will also provide industry with a powerful tool for inspection and fault finding.

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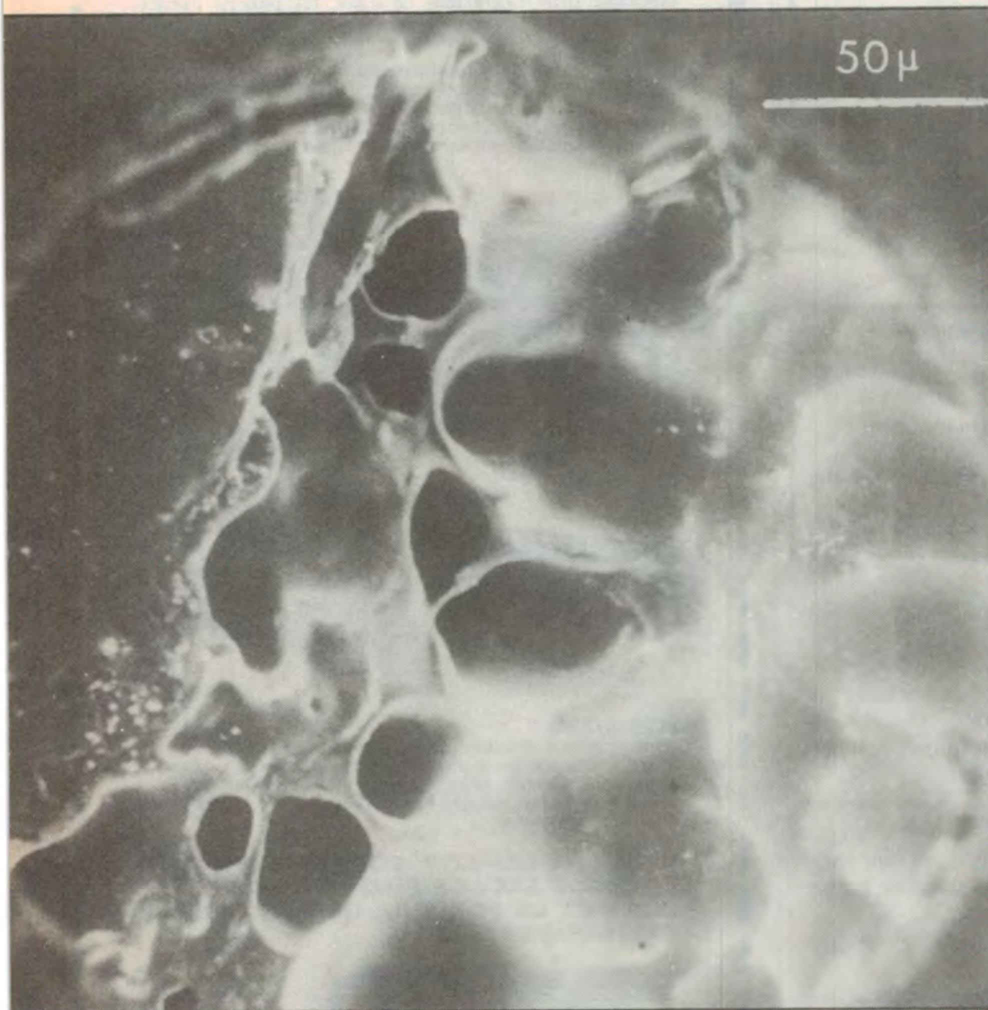


Scanning electron microscopy inherently suffers from the disadvantages that living specimens cannot be placed in the vacuum necessary for transmission of the electron beam, and the beam burns anything placed under it. A technique developed at Bristol University now permits electron micrographs such as this to be obtained. It shows a cross-section of a hydrated leaf with full retention of tissue fluid, indicated by intact domed surfaces of cells.

A desire to see structure, forms and morphology at microscopic scales is inherent to the curiosity of mankind. It was the driving force that led Anthony van Lee Hoek, a Dutch clockmaker, to devise a compound light microscope. Nowadays, of course, much larger magnifications can be obtained by electron microscopes. Yet optical microscopy still has a powerful advantage over electron microscopy: it can be performed on living matter without destroying it.

It is interesting to note that in 1926, when the US physicist Leo Szilard suggested to the British engineer Dennis Gabor that an electron microscope might be made by assembling electron lenses, Gabor (later the inventor of the hologram and winner of a Nobel prize in physics) rejected the idea and pointed out that living specimens cannot be placed in a vacuum, which is essential for electron beam optics, and that energy in the focused electron beam would burn and destroy anything placed under it.

In the event, the first such microscope was built by the German scientists Max Knoll and Ernst Ruska in 1931. Advances since then in many branches of materials science, biology and medicine can be attributed to the use of electron microscopy. This has been duly acknowledged by the fact that Ernst Ruska shared a 1986 Nobel prize in physics, so it is appropriate now to review how far electron microscopy for biological and other difficult materials has progressed.

With a modern, commercially available *transmission* electron microscope (the type developed by Ruska) we can visualise features and structures of only a few nanometres (10^{-9} metres). However, such an instrument requires the specimen to be thin, because an image is obtained by passing electrons through it. This means that surface features of a thick, three-dimensional object cannot be examined very well.

Additionally, because the specimens have to be very thin, it is extremely laborious to obtain three-dimensional information.

These disadvantages are overcome by the *scanning* electron microscope, a

type of instrument first built by the German physicist Manfred von Ardenne in 1938.

Depth of focus

The first commercial scanning electron microscope was made available in 1965 by Cambridge Instruments, a British firm near Cambridge. In this kind of microscope an extremely small, focused spot of electrons is made to fall on the surface of a specimen and scan across it in a "raster", just as in an ordinary television tube.

The electrons interact with the specimen and release secondary electrons from near the surface. (Some of the primary, incident electrons are absorbed within the specimen, while some are bounced back out of the surface; more about this back-scattering later). Emitted secondary electrons yield information about the surface topography.

In a conventional scanning electron microscope these secondary electrons are collected, point by point, and used to build a picture.

Deeper surface features of thick specimens can be imaged in a scanning electron microscope because the depth of focus is much greater than that in an optical microscope, so the technique gives a much more vivid impression of three-dimensionality. It displays morphological and topological features at much higher magnifications than in an optical microscope.

Because the instrument is relatively easy to use and can be combined with other analytical techniques, it has become enormously popular. Magnifications available with modern instruments are only slightly less than those possible in a transmission electron microscope.

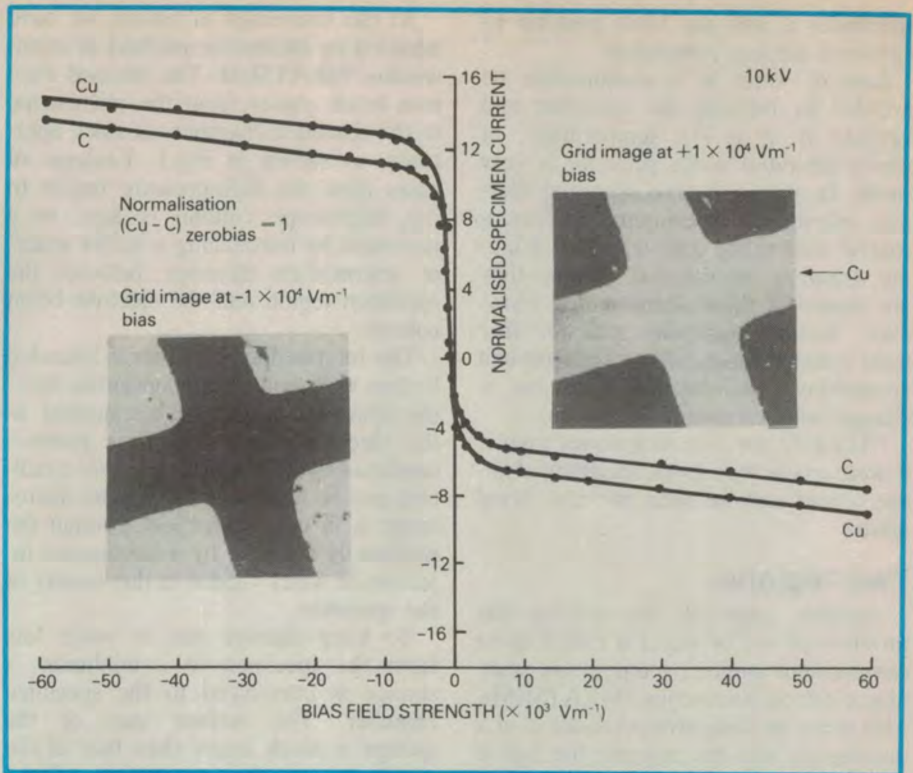


Fig.2: Variation of "normalised" specimen current with field bias strength for 10kV accelerated primary electrons. Normalisation is on the basis that the difference between the specimen current from copper and carbon at zero field strength is unity.

When it comes to viewing living matter, scanning electron microscopy has the same serious drawbacks as those pointed out by Dennis Gabor. Therefore we must be able to keep specimens in a microscope in a fully hydrated state, without loss of water. That is to say, they must be kept as near as possible to a living state.

In any electron microscope, a well-focused beam of high-energy electrons, necessary for imaging, has to be produced and kept in a high vacuum. It cannot travel long distances in a high-pressure gaseous environment without being scattered by gas atoms or molecules and losing its energy, and a badly scattered beam cannot render high resolution. This is an obstacle to electron microscopy of biological material in its natural, hydrated state.

For these reasons specimens are, conventionally, deliberately dried out and made stable for viewing by using procedures such as chemical fixation, dehydration and fluid replacement. Additionally, for scanning electron microscopy, dehydrated specimens, which are generally poorly conducting, are coated with a thin conducting layer of gold or of an alloy of gold and palladium to avoid a build-up of charge, for detailed features on charged surfaces cannot be imaged well.

But these techniques cause a drastic change in interfacial tension forces, which in turn causes delicate biological structures to become distorted and even to collapse. In spite of the development of special techniques for preparing

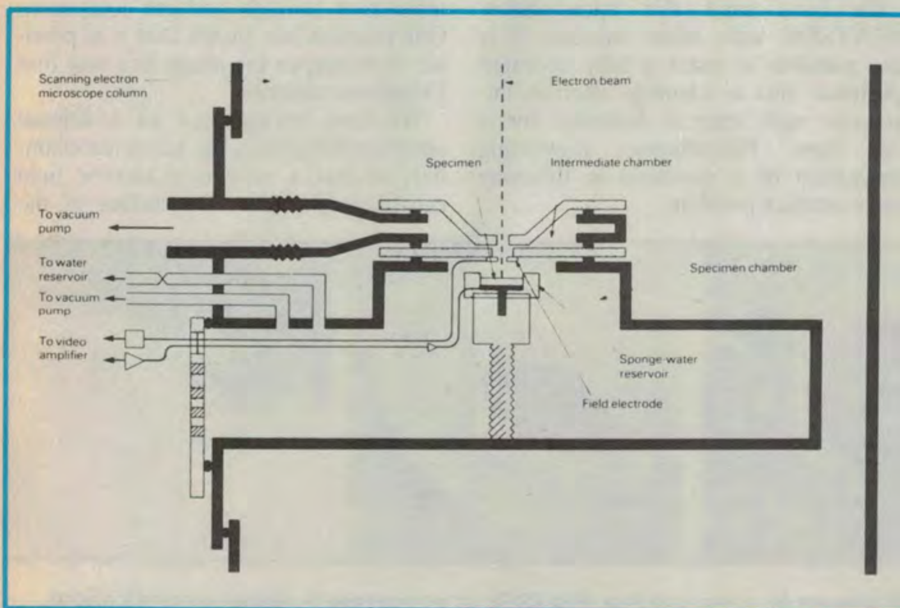


Fig.1: Schematic diagram of the apparatus used for MEATSEM.

specimens it has not been possible to eliminate damage completely.

Loss of water in a vacuum can be avoided by freezing the specimen and keeping it at a low temperature, at which saturated water pressure is very small. In so-called cryo scanning electron microscopy, specimens are frozen, coated with metal and transferred into the scanning microscope, where they are viewed at a low temperature. However, frozen specimens are not free from damage which takes place through anomalous expansion of water as it changes into ice crystals.

Obviously the cryo technique, even if it were made free from specimen damage, could not be used to view living matter.

Two regions

Another approach for solving the problem of loss of water is called moist environment ambient temperature scanning electron microscopy (MEATSEM). This relies on compartmentalisation of a microscope into two regions: the first is a high-vacuum region for electron beam production and electron lens optics; the second is a high-pressure region at room temperature, to surround the specimen and prevent it from losing fluid and gaseous constituents. (If a specimen is kept at saturated vapour pressure of water or 100 percent humidity it will remain wet, just like clothes hanging on a washing line on a humid day.)

Complete compartmentalisation can be achieved by using a window that is transparent to electrons but at the same time tough enough to maintain a high difference of pressure between the two compartments. This approach has the drawback that window materials scatter the electron beam badly. To keep scattering down to feasible levels the window has to be extremely thin, which means it is extremely fragile. Reliable windows with an acceptable loss of resolution are difficult to make.

At the University of Bristol we have adopted an alternative method of open-window MEATSEM. The focused electron beam passes from the microscope to the specimen chamber *via* small apertures, as shown in Fig.1. Leakage of gases from the high-pressure region to the microscope column is kept to a minimum by introducing a buffer space, or intermediate chamber, between the specimen region and the electron beam column.

The intermediate chamber is bounded by two walls containing concentric limiting apertures in the planes normal to the electron beam, and it is pumped continuously so that the pressure gradients can be maintained while the microscope is in use. Water lost through the window is replaced by a continuous injection of water vapour in the vicinity of the specimen.

To keep damage due to water loss from the specimen to a minimum, a sponge is introduced to the specimen chamber. The surface area of the sponge is much larger than that of the specimen, so the proportion of the water lost from the specimen to the total loss of water is very small.

Scattering of the electron beam in this arrangement depends largely upon the pressure in the specimen compartment and how far the electron beam travels through the high pressure to reach the specimen. Pressure in the specimen chamber is kept at the saturated water vapour pressure, at near to room temperature. With careful design of the apparatus the scattering of the primary beam can be kept down to give reasonable resolution.

We have used this open-window MEATSEM with some success: it is now possible to insert a fully hydrated specimen into a scanning electron microscope and keep it hydrated for a long time. Nevertheless, preventing desiccation of a specimen in this way poses another problem.

Additional electrons

Forming an image under these conditions presents formidable difficulties. The conventional technique of constructing an image by secondary emitted electrons does not work, because secondary electrons, primary electrons and back-scattered electrons ionise water or gas molecules close to the specimen and produce additional electrons. These electrons, which do not carry any information about the specimen surface, have a similar energy range to that of the secondary electrons emitted from the specimen, so they cannot be separated easily from the secondary electrons released from the specimen surface. Without such separation, there is a severe deterioration of the secondary emitted image.

Back-scattered electrons (deflected primary electrons from beneath the specimen) also carry image information. Because they are scattered from a larger volume of specimen, the resolution achievable by their use is not as good as that obtainable by using secondary electrons. Further deterioration in the resolution is also likely because the low-energy of back-scattered electrons means that they cannot be separated easily from spurious electrons.

Interaction of the primary electron beam with the specimen creates a charge which, in turn, generates a minute current in the specimen. The point-to-point variation of this current with scanning of the beam can be made use of for image generation. With a wet specimen the current can be collected without any metal coating. A specimen-current image is also susceptible to deterioration through ambient ionisation. Our research has shown that it is possible to resharpen the image in a way that I shall now describe.

We have incorporated an additional annular electrode in the specimen chamber, so that a substantial electric field can be produced at the surface of the

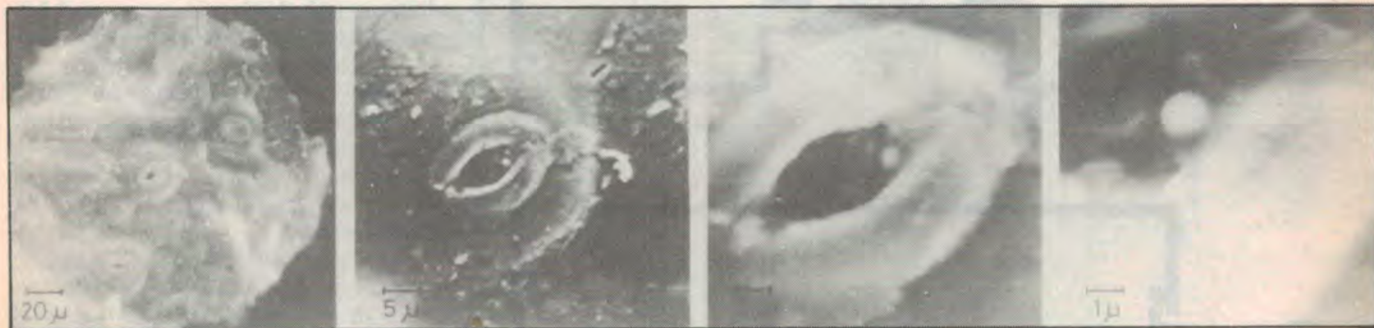


Fig.3: Stomata, or breathing pores of a leaf, viewed by open-window MEATSEM at successively higher magnifications. These eventually reveal a tubular structure with a slit within a stoma.

specimen. The field has a considerable effect on the specimen current, shown in Fig.2.

The images are of copper grid bars on a carbon surface, and the curves represent variations of specimen current with the strength of electric field from copper and carbon.

It is thought that the electric field helps to conduct the excess charged carriers, produced by the interaction of primary, back-scattered and secondary electrons, through the gases, which enhances the contrast in the specimen-current image.

Application of the field changes the magnitude of the specimen current rapidly at first and then more slowly as one or other plateau in the curve is reached. The image quality and contrast is re-established with the magnitude of the field applied. It is also possible to invert the image contrast by changing the direction of the imposed field.

The curves shown for copper and carbon indicate that the contributions to the current by both the back-scattered electrons and the secondary emitted electrons are recovered to a great extent in the plateau regions. So, once the specimen current reaches a plateau, the contrast, sharpness and resolution of the specimen-current image of an object under high pressure are substantially recovered. The image is comparable in quality to the conventional, secondary emissive image of a similar object in a high vacuum.

A series of pictures in Fig.3 shows recovered images of stomata of a fully hydrated leaf, at roughly 16°C, at various magnifications.

Stomata are breathing pores of a leaf, which automatically close and open to regulate exchange of water vapour between the leaf and the air. They are therefore suitable specimens to study by MEATSEM on a fully hydrated leaf.

The larger picture is a cross-sectional view of the same hydrated leaf, showing cells in between the cutical (outer) layers of the leaf. There are two distinct layers: one layer contains holes; in the other, denser layer the cells have intact upper domed surfaces indicating preservation of tissue fluid.

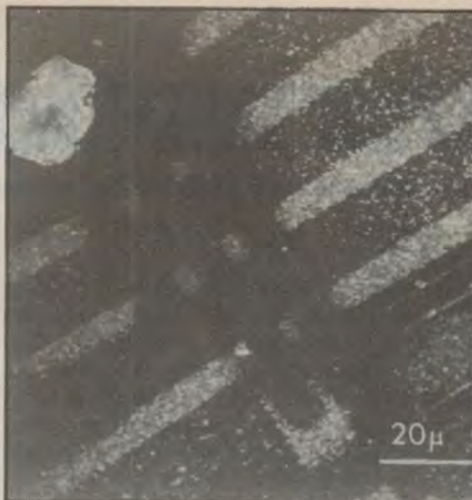
Fully hydrated internal tissue cells of animals can be imaged, too. The resolution obtained so far is limited by factors not directly related to the principle of the technique, while difficulties stem from the fact that the current from a typical uncoated "wet" biological specimen is smaller.

Results indicate that we may well see rapid progress in scanning electron mi-

croscopy of hydrated biological materials. In turn this will open up means of realistically assessing deterioration from other causes, such as radiation damage due to incident electron and heat produced by the interaction of electrons with a living specimen.

Optimistically, we may expect other advances such as low-voltage scanning electron microscopy combined with MEATSEM , which may well enable us to view live matter *without* inevitably killing the specimen. This will not only fulfil a long standing dream of mankind but also provide a tool for observing the dynamics of life at high magnification.

MEATSEM has led to a solution of another long-standing problem in scanning electron microscopy. I have already mentioned that insulating, semiconducting and poorly conducting materials are difficult to image, unless coated in a scanning electron beam instrument because of charge build-up on the surface of the specimen. It alters the trajectories of primary and secondary (emitted) electrons and the process of emission of secondary electrons; this grossly distorts the image and is also accompanied by loss of details and resolution. Charging can also bring about electric breakdown of the material, which is particularly serious in examining semiconductor chips containing circuits and active electronic devices.



Left: View of part of a circuit on a semiconductor wafer, showing charging effects; "spread" in the darker area is due to charging. Right: A view of the same circuit after charge neutralisation.

With certain modifications of the open-window MEATSEM, a charge neutralisation mechanism can be employed to reduce or eliminate charge build-up on a surface. The final illustration shows before-and-after images of a circuit on a semiconductor wafer. The improvement was achieved by charge neutralisation.

Potentially, the technique is a powerful tool for inspection and fault detection, and promises to have other industrial uses. Cambridge Instruments, who built the first scanning electron microscope, may well be the first company to make the MEATSEM and its associated techniques available commercially.

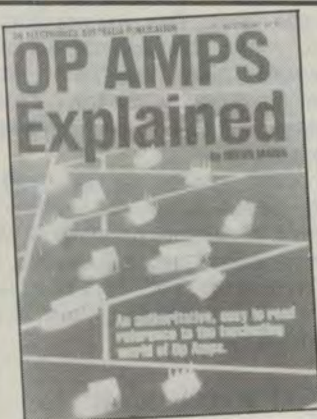
Radio *Continued from page 80*

or a metal water pipe which travels underground at some point. The soil should be reasonably conductive, otherwise it will be difficult to obtain a good connection. However, if this is not the case, there is probably not much that you can do about it anyway.

A few notes with regard to operation of a regenerative radio are perhaps in order here, as this type of receiver is something of an oddity these days. Begin with the regeneration control set about mid-range, and adjust the tuning control until the desired station can be heard. If the radio squeals, back off the regeneration until it stops. Conversely, if you don't hear anything at all, or if it is very weak, advance the regeneration control until *just before* the onset of oscillation.

Best performance is achieved when the radio is just on the verge of (but not quite) oscillating. If the volume is too loud, do not back-off the regeneration, as this will reduce the selectivity and introduce interference from other stations. Use the volume control instead. The oscillation may not always be audible, but instead may manifest itself as severe distortion of the audio output.

Have fun with your new radio!



Look inside almost any piece of modern electronic equipment (even things that are normally "digital") and you're likely to find op amps amplifying, filtering, shaping, clipping, detecting level changes or otherwise processing signals.

Without op amps, we'd still be in the electronic dark ages. And without sound basic understanding of the way they work and the way they're used, you probably won't get very far in today's world of electronics.

This book can be your guide and reference. It is authoritative, but at the same time very accessible and easy to read. The author has really boiled down the concepts of op-amp operation to make them understandable.

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