Is EM dead?

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Journal of Cell Science 126, 4545–4552
© 2013. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.124123

Summary

Since electron microscopy (EM) first appeared in the 1930s, it has held centre stage as the primary tool for the exploration of biological structure. Yet, with the recent developments of light microscopy techniques that overcome the limitations imposed by the diffraction boundary, the question arises as to whether the importance of EM in on the wane. This Commentary describes some of the pioneering studies that have shaped our understanding of cell structure. These include the development of cryo-EM techniques that have given researchers the ability to capture images of native structures and at the molecular level. It also describes how a number of recent developments significantly increase the ability of EM to visualise biological systems across a range of length scales, and in 3D, ensuring that EM will remain at the forefront of biology research for the foreseeable future.

Key words: Electron microscopy, EM, Scanning electron microscopy, SEM, Transmission electron microscopy, TEM

Introduction

For scientists using electron microscopy (EM) in their everyday research, the title of this Commentary might provoke a strong reaction. Yet for those working with light microscopes and the possibilities they now offer for imaging biological systems beyond the restrictions of diffraction, it is perhaps a logical one. This exact question was asked during a Company of Biologists Workshop on super-resolution microscopy (see Meeting Report). The rationale for writing this article, therefore, is first, to describe how far EM has brought us in our understanding of biological structure and function and, second, to show that, along with the revolution in light microscopy technology, EM has also undergone a sea change in recent years, with several new methods that have significantly expanded its capabilities for biological samples at both ends of the length scale and in 3D.

Although the expanding range of light microscopy techniques allows for imaging *in vivo*, for the moment at least, they rely on the detection of photons from labels engineered into the living systems. Unlabelled surroundings still remain hidden or unresolvable with sufficient fidelity to allow membranes or compartments to be distinguished. So, although dynamic processes can be analysed, any information about the surrounding structural setting is missing. Therefore, despite advances in light microscopy, it is difficult to see how it can currently challenge EM for the structural analyses that still remain at the core of so much of our research into the workings of biological systems.

This Commentary shows how the electron microscope, since its first appearance more than 70 years ago, has occupied centre stage as the final arbiter of biological structure. It will also describe some of the more significant studies that have shaped our understanding in various aspects of cell biology. These developments continue, and a few of the more significant ones will be outlined to illustrate how imaging with electrons today is probably more important in life science research than at any other time in its history.

Where it all began

The extraordinary thesis and then postdoctoral work of Ernst Ruska in the late 1920s and early 1930s, together with Max Knoll in Berlin, saw the conception and construction of the first transmission electron microscope (Knoll and Ruska, 1932) (Fig. 1). The idea was based on Louis De Broglie's thesis of wave theory for particles, with the electron's short wavelength giving far higher resolution than was possible with light. Although the first EM pictures of eukaryotic cells are attributed to Keith Porter (Porter et al., 1945), it was Ernst Ruska's brother Helmut whose pioneering work in the field of infectology produced the first transmission electron microscopy (TEM) pictures of bacteria as well as viruses (Ruska et al., 1939). Imaging in these early days was limited to samples thin enough to allow the electrons through to the photographic plates located below; however, the technique was quickly adopted by biologists wanting to see beyond the limits of light microscopy. It was a simple technique that soon played a crucial role in viral research, e.g. showing important structural differences between the small pox and chicken pox viruses (Van Rooyen and Scott, 1948; Nagler and Rake, 1948) (Fig. 2). What began as rudimentary images of viruses and bacteria soon expanded to include the thin periphery of cells – which were grown onto holders inserted directly into the microscope – that had first been chemically fixed and then dried to resist the vacuum and damaging effects of the electron beam.

Explorations of the cell's internal structure were not realised until the development of thin sectioning methods (Newman et al., 1949) and the use of plastics into which the samples could be embedded (Porter and Blum, 1953). The use of glass knives, with their perfect cutting edge, in ultramicrotomy, now meant that thick structures such as cells and tissues could be sectioned thin

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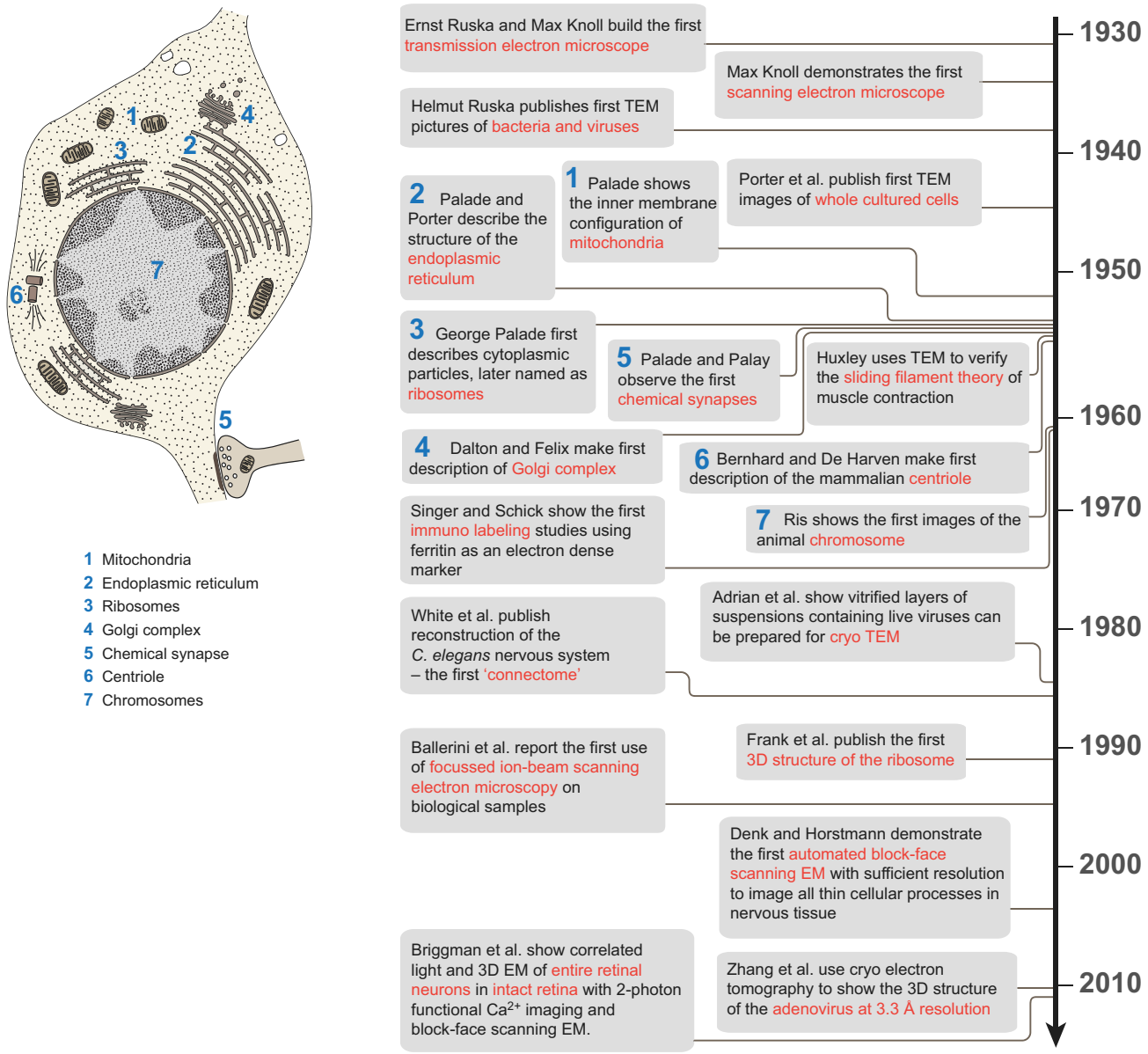

Fig. 1. Key milestones in the continuing development of EM for biologists. The figure highlights important discoveries of cell structure made by using EM.

enough (100 nm) for the electron beam to traverse. At that point, the large body of histology-staining techniques from light microscopy could be tested for EM and, soon, a number of preparation methods was born. These initial studies paved the way for a significant collection of papers exploring the ultrastructure and organisation of cells and tissues – way beyond the reach of light microscopy – that now form the basis of our understanding of cell and tissue ultrastructure.

Early studies began with detailed descriptions of the organelles (Fig. 1). Mitochondria, for example, had been studied first in whole-cell TEM imaging but later, in 1952, George Palade (winner of the Nobel Prize in 1974) showed their inner membrane configurations (Palade, 1952). Much later, this description was further elaborated using electron tomography (Perkins and Frey, 2000), whereby the sample is tilted in the electron beam and imaged at many different angles from which its 3D structure can be constructed. Two years after his first description, Palade – working with Keith Porter – made detailed descriptions of the structure they termed endoplasmic reticulum (Palade and Porter, 1954), an organelle that had been initially observed in 1945 (Porter et al., 1945). Around the same time, the Golgi complex was receiving much attention, and in 1954 Albert Dalton and Marie Felix published the first of three papers that described its organisation (Dalton and Felix, 1954). The plastic-embedding and thin-sectioning approach had quickly become the mainstay of any analysis of the cell structure that required high resolution; and this was elegantly demonstrated by Hugh Huxley in his Nobel Prize-winning studies on the sliding filament theory of muscle contraction, in which he showed the different filaments in muscles with TEM micrographs of longitudinally sectioned myofibrils (Huxley, 1957).

Much of this early work on cell structure had used brain tissue. The first descriptions of the ribosome, its attachment to the

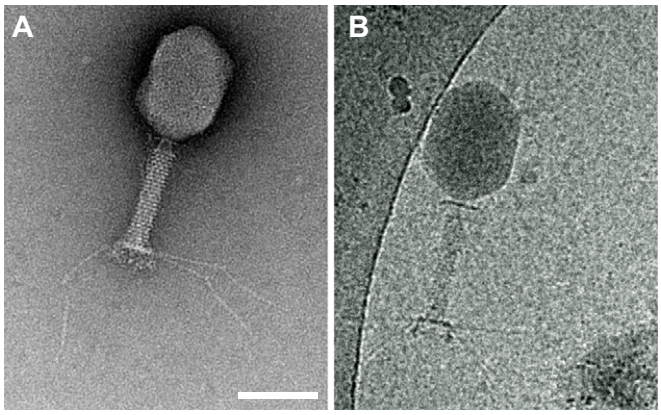

Fig. 2. TEM of whole T4 phage virus. (A) Electron micrograph of a T4 virus dried onto a thin support film, negatively stained with uranyl acetate and imaged at ambient temperature. The heavy-metal precipitate outlines the detailed structure of the virus, so that even the periodic molecular organisation can be distinguished. (B) Cryo transmission electron micrograph of whole, unstained T4 phage virus imaged in a vitreous layer of water. The virus was imaged with a low dose of electrons to avoid altering the sample. Scale bar: 50 nm (both images are at the same scale). Images courtesy of Davide Demurtas, Electron Microscopy Centre, EPFL.

endoplasmic reticulum and its aggregations forming the large granular body known as the Nissl body, were shown in neurons (Palay and Palade, 1955). EM also proved decisive when cementing the neuron doctrine – when the gap between the two apposed synaptic membranes was resolved, it showed unequivocally the concept that the nervous system was made up of individual, discrete cells rather than an anatomical syncytium (Palade and Porter, 1954); later it was shown that the vesicles of neurotransmitters accumulated on one side of the synapse, providing structural proof of the unidirectionality of these connections (De Robertis and Bennett, 1955).

At this time of intense activity and discovery using TEM, scanning electron technology was only beginning to appear as a credible technique for imaging surface topography at high resolution. Max Knoll was the first to use a scanning electron beam for imaging (Knoll, 1935); it was not until much later in the 1960s and 1970s that the Cambridge laboratory of Sir Charles Oatley brought the method to the eyes of biologists (Fig. 3). Scientists from Oatley's laboratory produced the secondary electron detector that is still so widely used today (Everhart and Thornley, 1960), as well as the first microscopes with a second column through which ions can be focussed and scanned across samples to etch away surfaces – the first focused ion-beam scanning electron microscopes. This development also produced imaging strategies that used lower energy beams while still maintaining their precision. This meant improved imaging of the surface topography was possible with fewer electrons penetrating deep into the sample. However, the technique relies on surface conductance to avoid the accumulation of charge. So, similar to the TEM images at the time, improvements in resolution would only produce higher magnification views of the metals used to coat and stain the underlying biological material. From its inception, biologists using EM were acutely aware of the disruption that their fixatives and staining methods caused, and much of the interpretation of the images needed to account for these artefacts. The struggle was to produce images that were a good representation of a dynamic and often watery environment in which molecular processes were occurring on very short time scales.

Efforts to image unstained biological structures with transmitted electrons, rather than visualising the stains themselves, appeared in the early 1970s with different types of sample holders capable of maintaining living samples close to their native states while resisting the vacuum and the harsh electron beam. These included tiny chambers that sandwiched the sample, with windows above and below to allow electrons through, as well as chambers with small apertures and a continuous flow of humid air to maintain the water content (Parsons, 1974). None of these proved particularly successful owing to the poor resolution and destruction of the sample during imaging, so efforts soon centred on imaging frozen samples. However, for this method to work, freezing rates needed to be high to prevent ice crystal formation, with only a few electrons being used to reduce the damage to the specimen.

Where we are now

Ice crystals that appeared during slow freezing rates did not seem to cause a problem for frozen hydrated crystalline samples, but this was not applicable for any others. The field was changed dramatically, however, when the team of Jacques Dubochet at the European Molecular Biology Laboratory proved that thin films of water could be vitrified (Adrian et al., 1984). When combined with sufficiently cooled microscope stages that maintain resolution and keep the ice in an amorphous state with a very low dose of electrons, the way was opened for imaging true native structures at high resolution. This first began with images of viruses, vitrified in a thin layer of ice on a TEM grid by plunge freezing (Fig. 2), and was followed shortly after by the development of reconstruction techniques of symmetric particles that could reveal their true 3D shape. Then came the imaging of

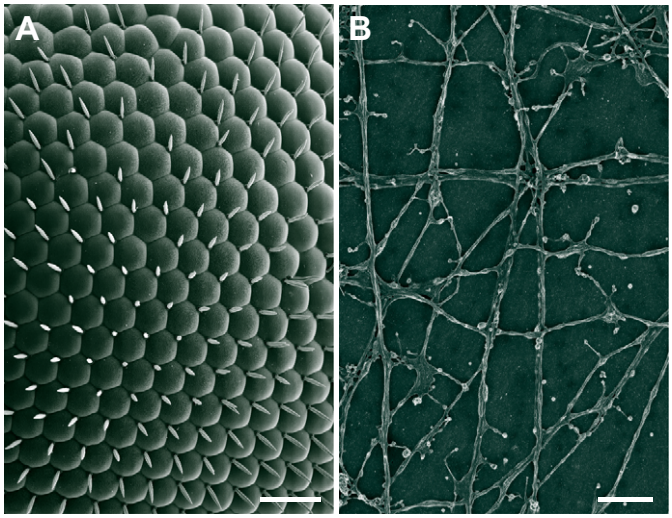

Fig. 3. SEM of two biological samples showing their surface topography. (A) SEM image of the eye of *Drosophila melanogaster*, and (B) neurites of axons and dendrites of cultured cortical neurons neurons growing on the surface of a Petri dish. Each sample was prepared using chemical fixation followed by dehydration, and then metal-coated with gold-palladium to provide conductance for the electron beam. Scale bars: 20 μm (A), 5 μm (B).

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non-symmetric structures, and notable amongst these was the first 3D model of the prokaryote ribosome from *Escherichia coli* at 40 Å resolution (Frank et al., 1991), from which the different functional parts could be elucidated. Since then, strategies for filtering the electron signal, based on their energy, have considerably improved the image quality of these low-contrast and fragile samples (Grimm et al., 1997). Most recently, the ribosome from the eukaryote trypanosome worm has been described at 4 Å resolution (Hashem et al., 2013). This gradual improvement in resolution has now reached an important point. The reporting of the human adenovirus structure at 3.6 Å resolution (Liu et al., 2010) and a metastable virus ready for cell entry at 3.3 Å (Zhang et al., 2010) shows how cryo-electron tomography can now challenge the resolution of crystallography. However, it can do so on complex, unstable atomic structures that could never be crystallised.

Initial imaging studies of cryo-preserved samples were only limited to small particles, such as viruses, because ice crystal damage on aqueous layers greater than a few micrometres was inevitable when plunging them into cryogens such as liquid ethane. Vitrification of thicker samples had to be achieved by the more vigorous approach of slamming samples between mirrored surfaces at cryo temperatures. Today, high-pressure freezers are even more effective in vitrifying greater volumes of sample by combining jets of liquid nitrogen simultaneously with very high pressures to slow the formation of ice crystals (McDonald and Auer, 2006). Vitrification of entire cells and tissues provides the means to preserve molecular structures in their native environment. With cryo-sectioning methods available to slice the sample thin enough for imaging with TEM, this is a significant turning point in structural biology (McDowall et al., 1983). It is now possible to visualise molecules within cells, rather than their purified version in a frozen buffer. The 3D reconstruction of desmosomal contacts between epidermal cells revealing the shape of the connecting cadherin molecules and their intercellular arrangement (Al-Amoudi et al., 2007) was an important demonstration of how it is possible to view molecules integrated in their natural surroundings. It also showed how data gathered from other sources, such as X-ray crystallography, can be matched with structures seen within cells to better understand the molecular arrangements.

Localising molecules to understand their position within the complex architecture of tissues and cells is perhaps where EM has held the strongest appeal amongst cell biologists and, particularly, those needing verification of their light microscopy data. Positioning molecules within a cell provides information about their function, and their arrangement is key to revealing how they can operate in such a highly coordinated manner. Chemical fixation methods, combined with non-specific heavy-metal stains that broadly delineate the large molecular complexes, are ideal for providing a broad range of information. These methods have also been widely used in combination with specific labelling methods, such as immunocytochemistry, or in correlative approaches combining light microscopy and EM, to pinpoint the positions of specific molecules. Therefore, the first studies that showed how electron-scattering particles, such as ferritin (Singer and Schick, 1961) or gold attached to antibodies (Faulk and Taylor, 1971), can reveal the position of components within cells have held a unique place as the gold standard of localisation studies. With high specificity and a suitable resolution to position molecules within a few nanometres, it is the most widely used technique for locating molecules amongst the other pieces of cellular machinery. There is a trade off with this technique, however, between having either good ultrastructure or a high level of immuno-labelling; but there are now many different variations, from using fragments of antibodies for better penetration to methods of cutting thin frozen sections of fixed material that are then brought to ambient temperatures for immuno-labelling. In principle, the methods can be divided into two categories: pre-embedding labelling, in which specific markers are added prior to embedding the samples in resin, and the post-embedding approach, in which the antibodies are applied directly to the thin sections. In both cases the level of fixation is crucial: too much, and the epitopes are tightly bound into their surroundings with little access to the antibodies; too little, and the ultrastructure is poorly preserved.

To circumvent this issue of getting access to the molecules in question, molecular approaches have recently produced genetically encoded tags for EM contrast. These are analogous to the fluorescent protein labelling for light microscopy that allows proteins to be specifically marked by genetic fusion. The first of these was horseradish peroxidase, which was used to label the secretory pathway (Connolly et al., 1994), but was limited to use under specific conditions, like high calcium concentrations. Recently, more versatile tags have appeared. The first is MiniSOG, a mini singlet oxygen generator comprising a fluorescent flavoprotein that produces singlet oxygen under blue light and can, therefore, polymerise diaminobenzidine (DAB) into an electron-dense precipitate (Shu et al., 2011). The second is a peroxidase called APEX, which can be used to precipitate DAB without the need for light (Martell et al., 2012).

The increased use of fluorescent technologies for imaging cellular events has also led to a number of different methodologies, whereby light microscopy is combined with EM. These provide structural correlates to the fluorescent signals that can also be captured from living samples. Serial section TEM, for example, was crucial for showing how the appearance of protrusions on the dendrites of neurons – termed dendritic spines and seen in the live adult mouse brain – corresponded to changes in synaptic connectivity (Trachtenberg et al., 2002). The GFP-expressing neurons were imaged live by using two-photon microscopy, and in the electron microscope by using immunocytochemistry to locate the fluorescent protein. More recently, however, studies have shown that these fluorescent neurites can be found in the EM sections – without the need for immunocytochemistry – by using the two-photon laser to burn reference marks into the fixed tissue (Bishop et al.; 2011, Maco et al., 2013). The fluorescent markers have also been shown to maintain a certain level of activity once embedded in the resin used for EM. This has been exploited by Kukulski and colleagues to show the translocation of macromolecules across cell membranes (Kukulski et al., 2012). By high-pressure freezing yeast cells at precise time intervals, the authors were able to study the shape changes of the membrane that occurred where they saw a fluorescent signal, therefore revealing structural details of the endocytic process.

Many correlative experiments image samples that are far thicker than can be included in a single EM section. Serial thin sectioning and TEM was, for many years, the only means of imaging large volumes and, in some cases, vast series have had to be manually cut and imaged. Recent years, however, have seen a

rapid evolution of 3D EM imaging technology, driven predominantly by the field of neuroscience.

From its early beginnings, EM has played a central role in the major discoveries of neuron and brain function. Neuroscientists were quick to develop the methods of serial-section TEM to formulate some of the basic principles of how nervous systems are wired together. The connectivity between neurons confers their basic functions, so the ability to analyse the structural arrangements of their contacts has been key. Amongst these efforts are some remarkable pioneering studies. Laboratories such as those of Alan Peters and Ed White, to name just two, developed the painstaking approach of manually sectioning pieces of neuronal tissue from various brain regions that were then photographed on negative plates and elegantly reconstructed using pen and paper to reveal their shape and connections (e.g. White and Rock, 1981; Peters, 2002).

Discovering the details of how neurons interconnect gives an insight into their function. A complete map of an entire nervous system would provide the basic connectivity blueprint through which an organism operates. So far, however, despite considerable interest, only one neural circuit has been mapped, an effort that took many years, demanding great skill and patience. This original connectome of *Caenorhabditis elegans* (White et al., 1986), however, formed by only 302 neurons, still represents a daunting mapping task for even modern-day serial-section TEM methods, with digital imaging and computing capabilities. Comparing this with the 20,000 neurons that comprise a single cortical column in the rat somatosensory cortex puts the scale of the problem into perspective.

Mapping the structure of every neuron and its connections through even the thinnest axon and dendrite requires a ‘synaptic resolution’ to distinguish all contacts, but the complete volume in which these components can be positioned in any orientation also requires reconstruction. Achieving this with serial-section TEM would require not only extraordinary skill and patience to avoid losing any of the sections and, therefore, the continuity of the wiring, but also some luck in avoiding hazards such as section folds and contamination on the sections that can obscure a clear view. These difficulties in producing very large series of sections have pushed the more recent development of new EM technologies for automatically capturing large and complete series of images, providing a huge impetus in the drive towards mapping whole neural circuits.

To overcome the problems faced by manually sectioning large samples, the group of Jeff Lichtman at Harvard University developed automated serial sectioning, whereby a thin plastic tape is used to collect sections immediately after they are sliced from the block face (Hayworth et al., 2006). This automated tape-collecting ultramicrotome (ATUM) method allows the cutting of many thousands of sections in series and from a larger block face than can normally be used with TEM imaging; the method also has a stability that allows thinner sections (30 nm) to be cut, increasing considerably the volumes that can be sampled. The resulting large line of sections is then imaged inside a scanning electron microscope, owing to the non-transparency of the collection tape to the electrons; the image is formed by either the ejected secondary electrons or the reflected backscattered ones.

This use of a scanning electron beam to capture information from serially sectioned material, however, has been simplified considerably by the use of an ultramicrotome placed inside the microscope chamber. The electron beam, instead of scanning a section, scans the surface of the sample block directly (Denk and Horstmann, 2004) (Fig. 4). For the level of automation alone, this serial block-face electron microscopy (SBEM) method has distinct advantages in being able to reliably collect uninterrupted series of many thousands of aligned images, with the diamond knife being able to skim off as little as 25 nm of resin after each image (Briggman et al., 2011). With reduced section loss, damage or contamination, this method also provides a means with which EM can now be applied to very large volumes of biological material, such as the complete thickness of a

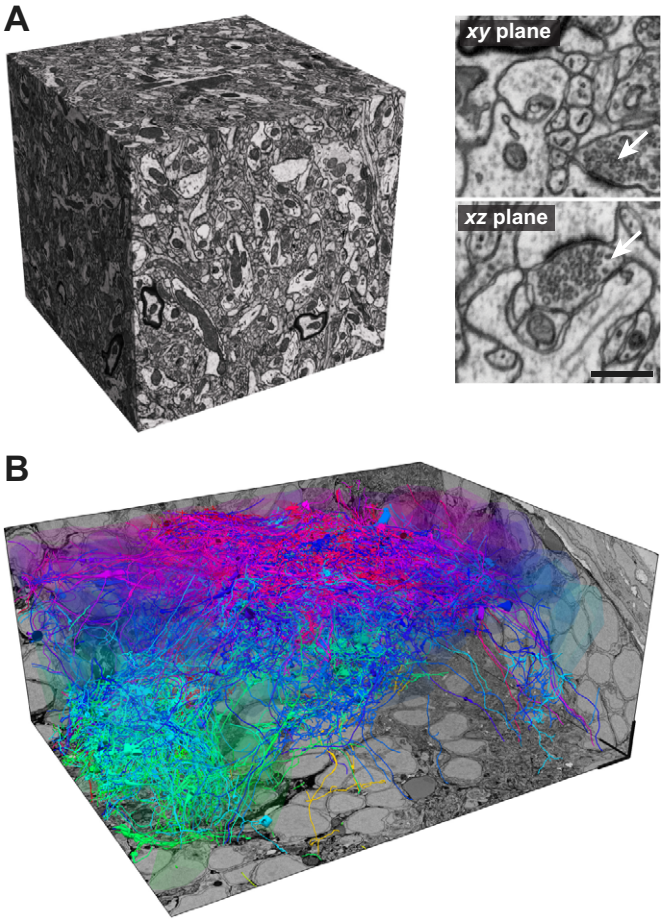

Fig. 4. SEM images using backscattered electron detection. (A) Serial FIB-SEM images of mouse adult cortex arranged into a 9×9×9 µm cube that was obtained by taking individual images at 5 nm pixel size with 5 nm spacing between each image. The isotropic quality of the image series means that structures can be viewed on different planes at the same resolution. Images on the right show the *x*, *y* plane and *x*, *z* plane (notice the similar appearance of the synaptic vesicles, arrows). (B) Manual reconstruction of 137 neurons in the olfactory bulb of a zebrafish larva (4 days post-fertilisation). EM images of the entire olfactory bulb were acquired using SBEM with a voxel size of 10 nm^3×10 nm^3×25 nm^3 (total volume of the stack shown is 71 × 45 × 38 µm). The skeletonised neurons represent the consensus of three manual reconstructions for each neuron. Neurons are colour-coded according to their soma location along the *z*-axis. The total length of reconstructed neurites is 31.4 mm. Images courtesy of Adrian Wanner, modified from Friedrich et al. (Friedrich et al., 2013). Scale bars: 0.5 µm (A), 5 µm (B).

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mammalian retina (Briggman et al., 2011) and pancreatic islets (Hoppa et al., 2012).

The once-fanciful idea of automatically imaging entire nervous systems has now become feasible. The only limitations, however, are given by the sensitivity of the block face to the electron beam. Increasing the electron dose increases the signal-to-noise ratio and improves the resolution; however, too high a dose affects the block surface and cutting stability. So the resolution is limited when consistent serial imaging is required. Nevertheless, the method can image many hundreds of thousands of cubic micrometres with voxel sizes of 20×20×25 nm – a resolution that is sufficient for visualising the thinnest neurites in mammalian brains.

The restriction in *z* resolution imposed by the sensitivity of the block face to the electron beam and the mechanical cutting is overcome when, instead of a diamond knife to cut away the layers, a beam of gallium ions, directed parallel to the surface of the block, is used. The focused ion beam hits the sample, sputtering it away as either secondary ions or neutral atoms. Using high currents the beam can be scanned over the sample surface removing as little as a few nanometres (>3 nm), giving an improved *z* resolution (Knott et al., 2008) (Fig. 4). Any electron-beam-induced alterations during imaging do not affect this milling process. The advantage is better 3D resolution; however, consistency in milling can only be achieved on far smaller fields of view (maximum 20×20 μm) with optimal resolution (4–5 nm per pixel). Nevertheless, focussed ion beam scanning electron microscopy (FIB-SEM) consistently captures images through many thousands of cubic micrometres at isotropic resolution, and the quality of the image data allows computer algorithms to automatically segment structures and reconstruct features (Kreshuk et al., 2011; Straehle et al., 2011).

The use of ion-beam milling is not just limited to gathering serial images of biological samples. Its accuracy in milling cryo-fixed material without the unavoidable cutting artefacts left by diamond knives has been exploited in the preparation of vitrified sections for TEM (Wang et al., 2012; Rigort et al., 2012). Although only small pieces can be prepared, this technique can also be combined with light microscopy data to select regions of interest (Rigort et al., 2010). This combination now offers the best opportunity for imaging molecular structures in the cell at specific moments at high resolution, based on the temporal information from light microscopy.

What the future holds

Considering the technological advances in EM that have become available to biologists during the last decade it would be foolish to try and make predications for the next. However, very recent developments could give some indications as to what might lie ahead.

The speed and accuracy with which images are captured are continually improving. However, despite the predominance of charge-coupled devices (CCDs) for TEM imaging, many of the high-resolution studies still opt for argentic films, because the dynamic range and resolution are considered superior. Yet the resolution that is routinely achieved without averaging still does not reach its physical limit, and one frontier that is moving quickly is the development of detectors that have larger numbers of pixels and better quantum detection efficiency. Better efficiency with less noise would reduce the amount of averaging currently required to improve the resolution. The number of sample particles needed for a high-resolution structure is huge [31,815 particles were photographed using 1350 negative films to acquire the structure of the adenovirus (Liu et al., 2010)], and acquiring these images is time consuming. Furthermore, the electron beam also causes the sample to move, adding further image aberrations. These problems, perhaps, explain why the number of complexes whose structure was solved by using EM tomography is low, and far lower than that obtained by using X-ray crystallography. For this reason, there is now a move away from the indirect method of electron detection, whereby electrons collide with scintillators producing photons that are recorded, towards direct detection systems using complementary metal-oxide-semiconductor (CMOS) devices. These show better efficiency and their speed allows for computational corrections of beam-induced movements (Grigorieff, 2013).

Another recent improvement to the imaging quality of cryo-samples has been the introduction of phase-plate technology. Phase plates act as spatial filters that modulate the diffracted electron waves, which improves the contrast of the cyro-samples. Although these plates have been around for many years, only recently have different types become available for use in biology (Nagayama and Danev, 2009).

At the other end of the EM length scale, the revolution regarding the volume that can be imaged and, particularly, the block-face scanning approaches described above, has extended the ability for collecting volumetric data at resolutions that are sufficient to visualise all cell membranes and large stained densities. These methods give unparalleled views of cell and tissue architecture. However, the acquisition of large datasets that incorporate, for example, complete neural circuits in mammalian brains, would take many days, even weeks. To illustrate this, imaging a field of view of 1×1 mm at 20 nm pixel size (*x*,*y* resolution) for a total depth of 1 mm by using 25-nm steps would require an acquisition time of 3 years if each pixel was scanned for 1 microsecond. Although the dimensions are feasible using the SBEM method, the length of time is prohibitive. Likewise, for a considerably smaller volume imaged at the higher resolution of 5 nm isotropic voxels by using FIB-SEM, a single cell of e.g. 20×20×20 μm in size, would take 7.4 days for imaging only (not including the milling time) if a suitable dwell time of 10 microseconds was used. However, the stability of these systems is not currently sufficient to leave them unattended for these long periods. So, despite the level of automation, the lengthy acquisition times for sizeable samples still present technical problems.

As a way to counter this issue, quicker imaging solutions are already appearing. The use of a fast camera array in a TEM has shown that final stitched images of 80k×120k pixels can be acquired ten times faster than using other commercially available systems (Bock et al., 2011). This method has the advantage of optimal *x*,*y* resolution (2 nm); however, the *z* resolution is still limited by the section thickness. Improvements to the imaging speed in scanning microscopy are also now available, with electron columns now capable of producing far higher doses. A radically new approach has also seen the appearance of a microscope prototype from Zeiss, whereby 61 parallel electron beams, rather than just one, can be directed at a sample simultaneously.

These opportunities of imaging large volumes create many terabytes of images from which only a fraction of the data can be extracted. Manual analysis methods are slow. For example,

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tracing the axons and dendrites within serial images of neural tissue can only be reliably achieved manually, with one person able to follow ~1 mm of axon in 5 hours. One mm^3 of mammalian cortex, containing about 4 km of axons, would therefore take 2283 years to map. As this is currently the only effective method to uncover the circuitry of the brain, the strategy to minimise the time has been to use cohorts of tracers, either as paid volunteers or even as participants in crowdsourced projects (e.g. www.eyewire.org) (Briggman et al., 2011; Helmstaedter et al., 2013). Automated segmentation algorithms are needed and, although not currently useable on any of the large-scale datasets, they are improving and, for the moment at least, can recognise and reconstruct features, such as synapses, mitochondria and neurites in the relatively small volumes of isotropic image data acquired by FIB-SEM (Straehle et al., 2011; Kreshuk et al., 2011). Therefore, it is perhaps the automation of image analysis in which the greatest rewards can be achieved in the future, with the extraction of information on a far larger scale than can be achieved today.

Improvements in both imaging and sample preparation have been ongoing since EM first appeared, allowing us to explore further into cells and tissues. However, it is only since the computerisation of microscopes that their abilities have really been exploited to give more routine methods for larger fields of view, and more-accurate 3D imaging of structures and analysis at higher resolution. The improved level of automation and simplification of imaging routines is making EM more widely available and, perhaps, no longer the preserve of dedicated specialists. Despite these significant improvements, we are still some way from reaching the theoretical limits of resolution for electron imaging. Tomorrow's microscopes, therefore, are likely to have even higher capabilities than those available today, and we can look forward to even larger datasets, more advanced automation and better automated analysis. Although this is no proof that EM is not dead, the few studies of biological structure that were mentioned here were not possible with light microscopes.

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