feature article

Cryo-electron microscopy of biological nanostructures

Robert M. Glaeser

The imaging of unstained and fully hydrated biological specimens embedded in vitreous ice is leading to powerful advances in understanding the structural basis of biological phenomena.

Bob Glaeser is an emeritus professor of molecular and cell biology at the University of California, Berkeley, and a scientist in the life sciences division at Lawrence Berkeley National Laboratory.

A wry joke states that you can see a lot just by looking. But in biology, to "just *look at the thing*!"—as Richard Feynman put it—really is a powerful method for understanding how cells and molecules work. Scientific investigation in biology often involves testing hypotheses regarding which subcellular structures are responsible for a process, and there is hardly a better way to call a particular idea into question than to just see whether such a proposed structure even exists. At the same time, the observation of a previously unsuspected structure immediately calls for formulating hypotheses as to why such a structure is there and how it can work. Visual observation, arguably one of the most powerful tools in biological research, is thus used to achieve both goals of the scientific method: It can rule out previously reasonable hypotheses, and it can generate empirical data that lead to the formulation of new, testable hypotheses.

The empirical characterization of various structures and their spatial distributions in a cell can play yet a third role in biology, one whose time may finally be arriving. Observed structures can serve as the boundary conditions that must first be stipulated before one can solve mathematical equations, derived from the basic laws of physics and chemistry, that apply to both the static and dynamic properties of a living system. Until the physical sciences are able to account for emergent properties in systems as complex as living cells, empirical characterization of hierarchies of structure, as envisioned by Michael Polanyi,[1] is arguably the most rewarding way to put our understanding of biology on a quantitatively testable basis.

An especially beautiful example of how just looking at the thing can provide a molecular explanation of a long-standing biological puzzle has recently come from using the electron microscope to examine the structures involved in allocating chromosomes to daughter cells during cell division. The movement of chromosomes requires that each be attached to one end of a group of microtubules, which together form what is known as the mitotic spindle. The other ends of the microtubules are attached to one of two "poles," one for each of the two daughter cells. Just before a cell divides, the chromosomes are paired in such a way that one member of each pair is attached to a given pole. This much was known from light-microscope images, whose resolution is usually not better than 0.5 micrometer (figure 1a).

What was puzzling about this process, however, was that the microtubules shorten during the movement of chromosomes to their respective poles. How could it be that they shorten without detaching from one end or the other and thereby lose their ability to pull? Another, seemingly unrelated puzzle was the unreasonably bizarre way in which microtubules disassemble. Microtubules are made up of 13 parallel protofilaments, each a linearly repeating polymer of subunit proteins called tubulins. The shortening of microtubules occurs when the protofilaments first peel away from the axis of the microtubule, splaying (figure 1c) like the petals of an unfolding flower, until they eventually drop off. This mechanism of disassembly seemed weird at the time it was first observed.

What Nature may actually be up to here became immediately clear, however, when electron micrographs revealed a ring structure formed by a protein complex, called Dam1, that is known to be required for the attachment of chromosomes to spindle microtubules. In combination with fluorescence light microscopy, those images showed that the Dam1 rings (figure 1b) can slide along the microtubule but cannot slip past the flared end. The hypothesis is that additional linker proteins connect the chromosomes to the Dam1 rings. The flaring ends of the microtubules then push the rings toward the poles, dragging the chromosomes with them, as is pictured in figure 1d.

No sooner has a puzzle seemingly been solved at one level of structure, however, than new questions arise at yet a smaller scale. Why is it, for example, that the individual filaments of a microtubule splay instead of subunits simply dissociating from the end, one by one? To answer that question, one must look at microtubules, and individual tubulin molecules, at a much higher resolution. Viewing biological structures at subnanometer resolution, however, requires techniques of sample preparation and data collection that are capable of retaining the samples' native, hydrated structure within the vacuum of the electron microscope.[2]

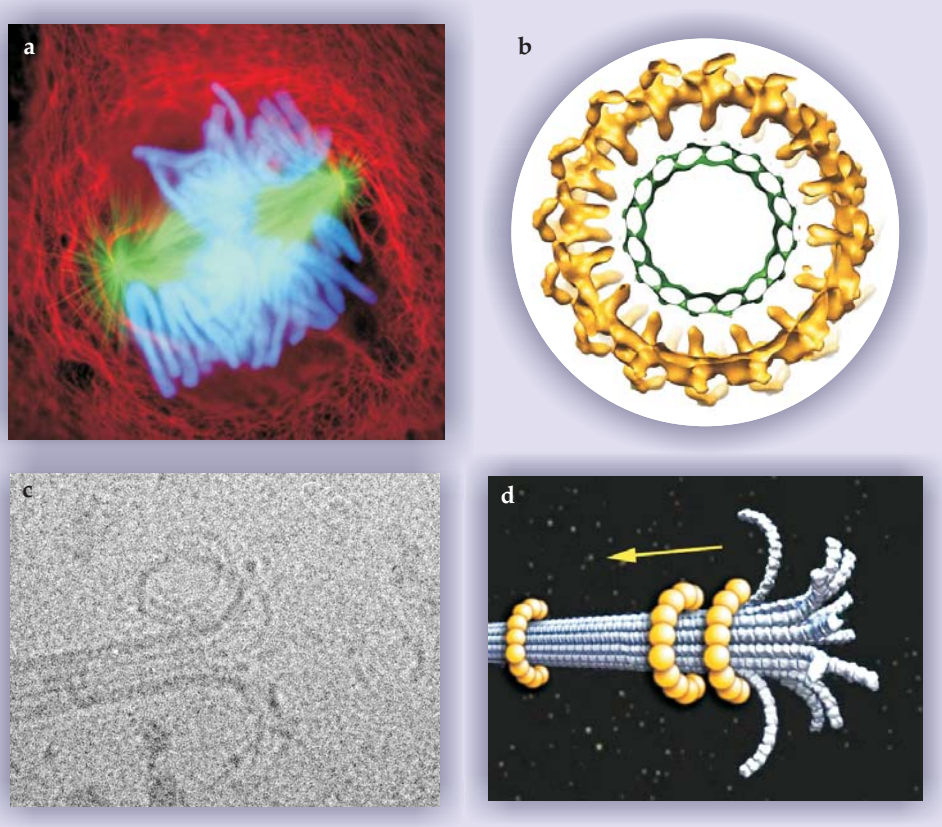

Figure 1. A complex structural system, involving key features at progressively smaller size scales, plays an essential role in the allocation of duplicate copies of the genome to daughter cells during cell division. **(a)** The mitotic spindle, readily visible in this light-microscope image, contains microtubules (green) that are attached at one end to a "pole" (centriole) and at the other end to one of the chromosomes (blue). Movement of chromosomes to the poles is accompanied by the shortening of the microtubules in the spindle. Keratin filaments, another component of the cytoskeleton, are shown in red. The width of individual chromosomes is about 2 μm. **(b)** Cryo-electron microscopy is helping to reveal the mechanism by which microtubules might be attached to chromosomes. One component of this attachment apparatus is the Dam1 complex, which Georjana Barnes, David Drubin, and colleagues discovered[11] makes rings (gold) that are able to slip along a microtubule (green). The diameter of the microtubule is about 30 nm, and the diameter of the Dam1 ring is about 60 nm. **(c)** When microtubules shorten, their constituent protofilaments peel away like the petals of an unfolding flower. **(d)** The bizarre method of shortening microtubules suggests that one function of the structure is to prevent the Dam1 rings from slipping off the disassembling ends. (Panel **a** courtesy of Conly Rieder, Wadsworth Center, Albany, New York; panels **b–d**, by Eva Nogales, University of California, Berkeley.)

Staying wet in a vacuum

But how is it possible to look at "wet"—that is, properly hydrated—structures in vacuum? The challenge of preparing fully hydrated specimens for electron microscopy is daunting because the sample must be observed in a high vacuum, due to the large cross sections for scattering electrons. In addition, the large scattering cross sections demand that the specimens themselves be very thin. Following early attempts to use either thin-window wet cells or differential pumping to keep specimens in a fully hydrated state, success was finally obtained by freezing samples in thin aqueous films and observing them at a temperature low enough to prevent sublimation of the ice. The demonstration that the structure of a frozen, hydrated protein crystal was preserved at much higher resolution than when the sample was prepared by traditional techniques involving dehydration and staining with heavy-metal salt, and the subsequent demonstration that the image contrast of such unstained specimens was much greater than most researchers had supposed it would be,[3] launched the branch of biological electron microscopy that is now called "cryo-EM." As described in the box on page 53, the subsequent addition of a simple procedure to vitrify the surrounding water during freezing to avoid crystallization—first achieved by Jacques Dubochet and his colleagues about 20 years ago[4]—provided a crucial breakthrough that enabled the current success of cryo-EM.

But one should be careful what one asks for: The development of a practical method to prepare unstained, hydrated specimens forced biophysicists to confront a couple of physical limitations that the early developers of electron microscopes had worried about from the start. In 1928, for example, Dennis Gabor had rejected Leo Szilard's suggestion to make a microscope based on electron waves with the response, "What is the use of it? Everything under the electron beam would burn to a cinder!" Even when cryogenic specimen temperatures were shown to increase the electron exposures that biological structures could tolerate by a factor of 5–7, the higher exposures were still about four orders of magnitude short of what is required to form statistically well-defined images at a resolution of 3 Å. Even at a much lower resolution, statistical fluctuations—shot noise—in the number of electrons per pixel are a major limitation if the electron exposure is kept low enough that features of interest are not destroyed.

The statistical limitations on image definition can only be overcome by signal averaging. The required spatial

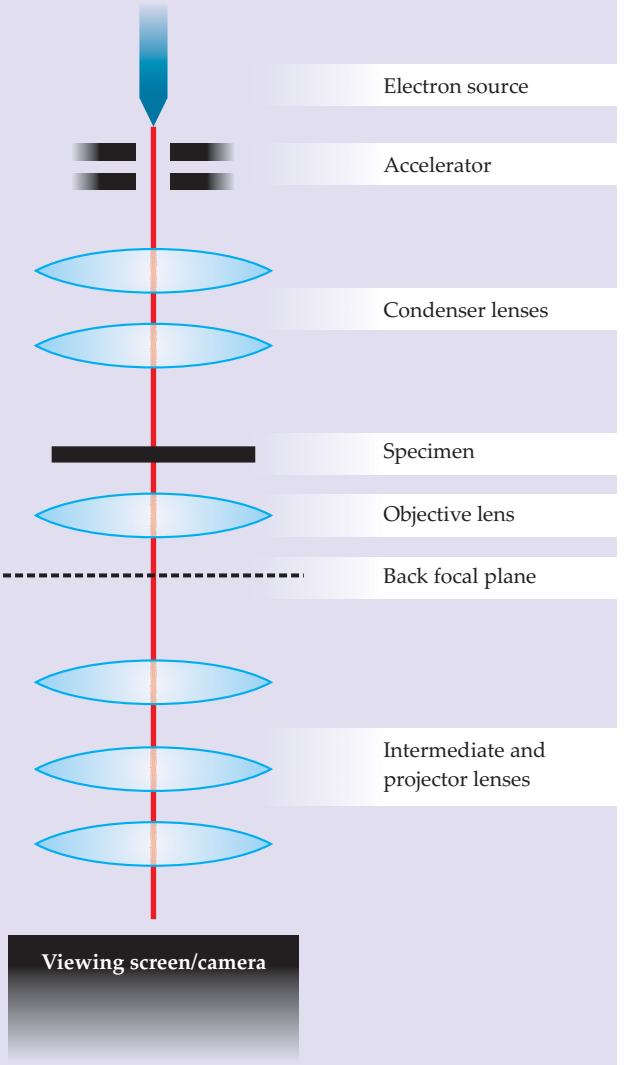

Figure 2. In an electron microscope, accelerated electrons are focused by condenser lenses onto the specimen, and the incident beam can be described to a good approximation as a plane wave. The electron wavefunction exiting the specimen forms a Fraunhofer diffraction pattern in the back focal plane of the objective lens, where the scattered electrons are distributed radially and azimuthally while the unscattered electrons are focused onto the optical axis. The scattered and unscattered components of the electron wave are recombined in the image plane of the objective lens, the resulting image is further magnified by subsequent lenses, and the final beam intensity is viewed on a fluorescent screen or recorded with a film or CCD camera. However, biological samples produce little variation in the intensity of the transmitted electrons; instead, they primarily introduce spatial variations in the phase of the transmitted wavefunction. To generate contrast in the image intensity for such phase objects, one must either defocus the objective lens or insert a quarter-wave plate device, as is used in phase-contrast light microscopy.

averaging is easier to do with specimens that are prepared as thin crystals—all molecules have a common orientation and are at known positions relative to one another. Although rapid data collection with thin crystals still presents unsolved experimental challenges—primarily when such specimens are tilted to collect data for a three-dimensional reconstruction—persistent efforts have revealed the structures of some extraordinary protein molecules at a resolution approaching that of x-ray crystallography. The use of monolayer crystals is an especially natural way to study the native structure of membrane proteins, for example. That approach has been used successfully for bacteriorhodopsin, a light-driven proton pump; for a light-harvesting, "antenna" protein from chloroplasts; for the ion-channel protein that is activated by the neurotransmitter molecule acetylcholine; and for a protein that does double duty in the lens of mammalian eyes, serving as both a water channel and a cell-adhesion molecule. Many other membrane proteins have been studied at lower resolution, where features such as the alpha helix, a common structural element in folded proteins, have been resolved but not the polypeptide chain itself. Monolayer crystals of soluble proteins have also been studied by cryo-EM, and in the case of tubulin a structure has been obtained at a high enough resolution to build an atomic model.

Averaging of images is also possible for an ensemble of well-dispersed, individual copies of macromolecular complexes, such as the bacteriophage shown on the cover of this issue. Averaging images of individual particles, however, requires a vastly increased amount of computation because, unlike for a crystal, there is no prior information about molecular positions and orientations. A further disadvantage is that particles must be sufficiently large that one can align their images and assign their relative Euler angles with a high degree of accuracy. Estimates of the ultimate limits of aligning images of single particles nevertheless suggest that atomic resolution is attainable for proteins as small as the hemoglobin molecule.[5]

Contrast complications

The theoretical issue isn't relevant at this point, however, since the contrast level measured in high-resolution images usually remains a factor of 10 less than that of the beam after it is initially scattered by the specimen. While a fraction of the initial signal gets lost due to factors such as imperfect optics and detectors, variations in the image quality—which can be quite large over the field of a single image—must be due to other experimental complications such as specimen charging and beam-induced movement of the object during exposure. Even so, cryo-EM images of sufficiently large single (that is, noncrystallized) particles, such as icosahedral viruses, ribosomes, and the protein-folding machine GroEL, easily show molecular structure at the level of alpha helices but not yet at the level of the individual amino acids that make up the polypeptide chain.

Ice-embedded biological macromolecules are essentially "phase objects"—that is to say, information about the structure of such specimens is encoded mainly in spatial variations of the phase, rather than the intensity, of the electron wavefunction as it exits the specimen. The phase at each point in the exit wave of a suitably thin specimen (many biological specimens are thinner than a few tens of nanometers) is well-represented by the Wentzel-Kramers-Brillouin (WKB) approximation—by the line-integral or projection of the shielded Coulomb potentials for all atoms along a given ray. What is the physics involved that makes it possible to obtain

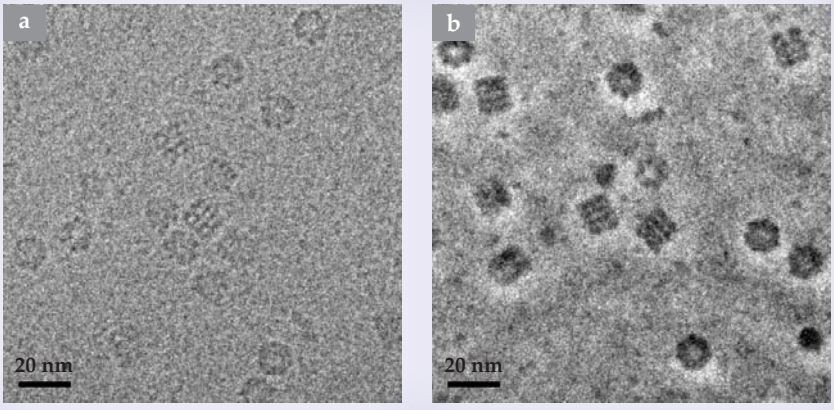

Figure 3. The contrast in cryo-electron microscopy images of biological macromolecules is much greater when a miniature device is used to apply a phase shift of 90° to the electrons that scatter in the sample. Unstained biological specimens alter the phase of the electrons but not the beam intensity. As in light microscopy, some image contrast is produced for such a pure phase object when the image is intentionally defocused. The addition of a phase-shifting quarter-wave plate behind the objective lens produces much greater contrast, however, and it does so without sacrificing high-resolution information, which gets corrupted with intentional defocus. **(a)** A cryo-EM image, with the objective lens defocused by about 2 μm, of GroEL, an 800-kilodalton protein complex that assists in the proper refolding of misfolded proteins. **(b)** An in-focus cryo-EM image of GroEL when a thin carbon film is used as a quarter-wave phase plate. (Images courtesy of Kuniaki Nagayama and Radostin Danev, Okazaki Institute for Integrative Bioscience, Okazaki, Japan.)

images of the information contained only in the phase but not in the intensity of the transmitted-electron wavefunction?

Imaging the phase

Deviations from a constant value of the phase across the exit face give rise to the far-field diffraction pattern, which can be seen by viewing the intensity distribution in the back focal plane of the objective lens downstream of the sample (figure 2). The unscattered component of the wave is focused onto the axis in the diffraction pattern, while the scattered wave is radially and azimuthally distributed in the pattern. What happens when the scattered and unscattered waves recombine to form an in-focus image is easiest to explain in the case of thin specimens, for which scattering can be described by the first Born approximation. Since there is a 90° phase shift for single-scattering events, the recombined waves add in quadrature. As a result, the image intensity is insensitive to variations in the relatively small values of the scattered amplitudes.

As Frits Zernike explained in his acceptance lecture for the 1953 Nobel Prize in Physics, awarded for his invention in the 1930s of the phase-contrast microscope, an optically perfect microscope lens produces a nearly exact, but magnified, copy of the intensity of the wave (whether light or electrons) that is transmitted through the specimen.[6] Transparent phase objects are thus all but invisible in a perfectly focused image—they show no contrast. But that physical reality, Zernike went on to say, has never stopped users of the light microscope from examining optically transparent objects (unstained, live cells, for example). Instead, light microscopists had found that all they need to do is simply defocus the objective lens by the right amount. Indeed, physical optics says that defocusing the microscope has the same effect as applying a 90° phase shift to the scattered wave, albeit only over a finite range of spatial frequencies. That additional phase shift causes the scattered wave to add constructively and destructively with the unscattered wave, and that interference introduces variations in the image intensity that are proportional to the amplitude of the scattered wave.

Similarly, if one chooses an appropriate amount of defocus, it is also possible for work to proceed in an electron microscope when using unstained cryospecimens. Misfocusing the microscope to generate the contrast that is needed to see an object involves, unfortunately, a bit of a Faustian bargain, for doing so severely degrades the image at higher resolution. Fortunately, much of the image corruption caused by defocusing can be compensated by computer processing a posteriori. Even so, some of the signal is irreversibly lost, and that loss becomes particularly severe at higher resolution.

A better way to image phase objects in the electron microscope would be to develop a device, just as Zernike did for the light microscope, that could apply a 90° phase shift to the electron wave in the objective lens's focal plane, where the Fraunhofer diffraction pattern forms. Since the scattered and unscattered components of the transmitted wave are separated in the diffraction pattern, one can imagine that it should be physically possible to selectively shift the phase of the scattered electrons relative to that of the unscattered electrons. The benefit of doing so was recognized by Hans Boersch, who in 1947 published three conceptual ways for producing electron-optical devices that would be the physical equivalents of Zernike's quarter-wave plate in the light microscope.[7]

Two of Boersch's ideas required the use of microfabrication techniques that would not be developed for decades to come, but one required only to cover the objective aperture's opening with a thin film, such as evaporated carbon, and to fabricate a small hole in the center for the unscattered electrons. In retrospect, the technology then available could not yet fabricate a hole small enough to provide phase contrast at low spatial frequencies. In addition, attempts to implement the idea in the 1970s and 1980s must have been limited by electrostatic charging of the carbon film due to the hydrocarbon contamination that builds up when an object is

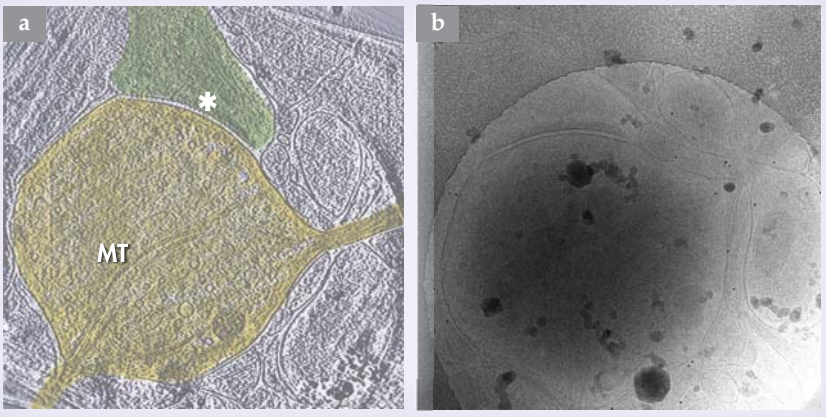

Figure 4. Cryo-EM tomography of whole cells—if they are prepared as suitably thin specimens—solves the problem of the overlap of subcellular features that occurs in single-projection images. Here, tomography has been used to obtain a three-dimensional image of a group of nerve cells. The microfabricated hole (large circle in panel **b**) in the support film on which the neurons have been cultured is 2 μm in diameter. **(a)** This tomographic slice includes a synapse, which is characterized by a dense molecular apparatus (indicated by an asterisk) on the inner surface of the membrane of the postsynaptic cell (shaded green). Many small vesicles, filled with neurotransmitter molecules, are present in the adjacent, enlarged body of the presynaptic cell (yellow). Microtubules (MT), shown coursing from the right center to the bottom left, fill the axon of the presynaptic neuron, where they serve as "railroad tracks" for motor molecules that carry cargo between the end of the axon and the main cell body. The microtubules likely also serve a structural role. Their walls, roughly 5 nm thick, provide an estimate of the resolution achieved in this tomographic reconstruction. **(b)** In contrast to a 2D tomographic slice, this 2D projection image of the same cell provides only an indistinct impression of most of the structural features in these cells, and all information is lost about the positions and sizes in the third dimension of those features that can still be identified. (Images courtesy of Vladan Lucic and Wolfgang Baumeister, Max Planck Institute of Biochemistry, Martinsried, Germany.)

hit by an intense electron beam.

The idea of using a thin carbon film as a quarter-wave plate for electrons was taken up again by Kuniaki Nagayama about 10 years ago. By heating the carbon film to about 200° to minimize hydrocarbon contamination, and by using modern focused-ion-beam technology to drill a hole with a radius of only 0.25 μm, his laboratory has recently produced spectacular results that seem certain to usher in a new era of biological electron microscopy.[8] As an example, the visibility of the GroEL protein complex is markedly improved when a phase plate, rather than image defocus, is used to provide phase contrast, as shown in figure 3. Although more contrast could be generated by defocusing the image much farther than the roughly 2 μm used in figure 3a, doing so comes at the expense of greater loss of signal at higher resolution.

The effort to use modern microfabrication tools to build an electrostatic phase plate of the type proposed by Boersch—a micrometer-sized, three-electrode device—has only begun more recently. The device, known as an einzel lens, consists of a planar electrode shielded above and below by grounded electrodes. Small, aligned holes in the centers of each of the three electrodes allow the unscattered electrons to pass through on axis, where they experience a phase shift due to the voltage applied to the central electrode. The scattered electrons, on the other hand, pass outside the device and thus experience no such phase shift. The advantage of the electrostatic phase plate is that none of the scattered electrons need be lost from the image. In addition, the magnitude of the phase-contrast effect can be readily manipulated by varying the voltage applied to the biased electrode. Although a proof of concept has recently been achieved in experiments with electrostatic phase plates, the device radius of greater than 2 μm is still about 10 times larger than it needs to be for the short-focal-length objective lenses found in modern, high-resolution electron microscopes. What remains to be seen is whether a suitable compromise can be struck between the difficulty of further miniaturizing the phase-contrast electrodes on the one hand, and the disadvantages of increasing the objective lens focal length on the other.

Cryo-EM tomography

A new problem, the overlapping of images of separate molecules, arises when electron microscopy is applied to whole cells rather than isolated components or thin cell sections. The cytoplasm of a living cell often consists of as much as 30% protein and other macromolecules, and thus images of many different, separate structures are superimposed in even the thinnest margins of a cell. The entire cell must therefore be imaged in 3D, and that can be done by tomography—the reconstruction of a 3D object from a series of images recorded over a range of angles that approximates, as well as possible, all directions of view.

When applied to rapidly frozen, unstained cells, EM tomography produces amazing results. Figure 4 shows an example of the complex structure that exists in just a thin slice cut out of a 3D tomogram, in the region of synaptic contact between two nerve cells.

The electron exposure required to image an object in 3D is no greater than what one normally uses to record a single 2D projection. The same total exposure has only to be fractionated into the different projections needed for a full tilt series. Of course, each of the projections is far noisier than if all the electrons were used to produce a single projection image. However, when the data are merged into a 3D image, the signals derived from each such projection add coherently while the noise adds in quadrature. As a consequence, any feature that could be imaged with adequate signal-to-noise ratio in a single projection can be imaged in 3D with the same S/N ratio.

Vitrifying the aqueous medium

Embedding biological specimens in a thin film of frozen, aqueous medium has many advantages for viewing their structures in the electron microscope. If one holds frozen specimens at a low enough temperature—typically around 110 K—the surrounding water does not measurably evaporate over several hours of experiments. As a result, the specimen is never subjected to the severe environmental change of becoming dehydrated, let alone the enormous interfacial forces that occur in air-drying. A collateral benefit is that the small molecular fragments produced by ionization and bond rupture tend to remain "caged" in locations that differ from those of the original structure by as little as 0.2 nm. Free radicals that are produced during radiolysis are also immobilized at low temperatures; thereby secondary reactions are suppressed that would otherwise further damage the structure. Detailed features of biological macromolecules can thus withstand electron exposures that are about 5–7 times greater than those tolerated by the same specimens when they are irradiated at room temperature, as was shown in the late 1970s by Kenneth Taylor and by Steven Hayward at the University of California, Berkeley.

It is crucial that the embedding water should solidify in a vitreous, amorphous state during freezing, as the nucleation and growth of ice crystals tends to steal water molecules that are otherwise needed for proper hydration of a biological macromolecule. Even more damaging, growing ice crystals tend to exclude solutes, especially those as large as protein molecules. The biological specimens thus rapidly become segregated into ever smaller volumes of not-yet-crystalline water as ice-crystal growth progresses. As the macromolecules become confined to the boundaries between different ice crystals, they aggregate and ultimately become deformed. Vitrification thus provides the greatest possible opportunity for the macromolecules to remain in their native, properly hydrated state.

Vitrification of liquid water is notoriously difficult to achieve, however. In protein crystals, the water content is relatively low, and the nucleation and growth of ice crystals does not usually occur when thin samples are plunged into liquid nitrogen. Unfortunately, that simple technique proved unsuccessful for well-dispersed, noncrystalline biological particles such as viruses. But as the laboratory of Jacques Dubochet discovered,[4] the freezing rate becomes fast enough to vitrify even such specimens when they are plunged into liquefied ethane. Unlike liquid nitrogen, liquid ethane does not make a film of vapor at the interface with the initially warm specimen. Importantly, the rather thick film of condensed ethane that coats the biological specimen is readily evaporated in the vacuum of the electron microscope. Since ethane evaporates at a temperature well below the glass-to-crystal phase-transition temperature of the vitrified ice, the coating of cryogen can be removed without undoing the benefit of vitrification.

Embedding samples in an air-dried film of glucose or other appropriate polar solvent has provided an alternative to embedding in vitreous ice and is especially effective for preparing thin crystalline specimens, such as the ones described in this article. Compared to vitrification, glucose embedding produces specimens that are much flatter (that is, more planar), which is essential when images are to be averaged over large, continuous areas. However, electron microscopy of such specimens is still performed at low temperatures to take advantage of the reduced radiation damage that occurs under cryogenic conditions. The glucose-embedding technique is not practical for single particles, though, because the density of glucose almost perfectly matches that of proteins, which makes it essentially impossible to see objects as small as individual macromolecules.

Cryo-EM tomography can nevertheless still benefit from the development of large-area, active-pixel-type detectors that are currently in development for high-energy physics experiments.[9] Such detectors promise a combination of reduced noise, high detection efficiency, and fast digital readout. Those features will allow more images to be taken in a tilt series and thereby achieve higher resolution in the tomographic reconstruction.

In addition, technology still needs to be developed to prepare very thin specimens from whole cells or even from whole tissues. Ideally, sample thicknesses should be no more than about 100 nm, a fraction of one mean free path for inelastic scattering; otherwise, too much of the signal may be lost in double or higher-order scattering events. Although the unwanted, inelastically scattered electrons can be removed by adding a commercially available energy filter to the electron microscope, the total exposure that a sample can tolerate is still limited by radiation damage. As a result, the loss of electrons due to inelastic scattering produces a progressive worsening of the electron shot noise in the image as one tries to use thicker, rather than thinner, samples.

Bypassing limitations

"Perhaps the human genius will contrive a way, quite different from the one we use now, to bypass these limitations which we now feel insurmountable." That quotation is attributed to Ernst Abbe, who in the 1870s was the first to explain image formation in a light microscope in terms of an inverse Fourier transform of the Fraunhofer diffraction pattern that exists in the back focal plane of the objective lens. The insurmountable limitation that Abbe spoke of is the fact that far-field diffraction sets "a limit to our vision which we cannot exceed." Ingenious though it may be, electron microscopy does not really qualify as bypassing the diffraction limitation since it gains its enormous improvement in resolution over light microscopy by simply using radiation of much shorter wavelength.

High-energy electron beams are much more than just a flux of extremely short-wavelength, focusable particles, however; they are also a flux of ionizing radiation. Thus there is a limit to how many electrons can be used to produce an image; beyond that limit the accumulated damage has too great an effect on the specimen. Unstained biological specimens are, unfortunately, among those whose structures are the most easily damaged by ionizing radiation. As a result, it is natural to ask whether inelastic scattering and ionization will forever limit the power of electron microscopy in biological research in the same way that it does today: Is it possible to overcome this limitation "which we now feel insurmountable"?

It is fun to identify some ideas that are in the air at the moment. The purpose in doing so is to encourage both a deeper investigation of them, and possibly to stimulate other ideas to be proposed, with the hope that the human genius

may one day improve even further the power that electron microscopy can have in biological research.

Waves, unlike classical charged particles, can be used to make measurements in which absorption—that is, inelastic scattering within the object—is suppressed. The trick is to arrange a way for destructive wave interference to decrease the probability of outcomes or measurements in which absorption occurs within the specimen. The technique has already been demonstrated experimentally for imaging with photons;[10] the question is whether "interaction-free imaging" can also be applied to electron microscopy.

In another research direction, ultrafast pulses in which each pulse contains one or a few electrons are currently being considered for electron microscopy of beam-sensitive specimens. Such pulses are useful for pump-and-probe experiments in which an object can be repeatedly excited so as to average the signals obtained over many cycles. But there is no physical reason to expect that they would reduce inelastic scattering (ionization) and the structural consequences that currently occur when electrons are emitted from a source at random times, but with the same mean time between electrons. But if the current typical exposures of 1000 or more electrons per square nanometer could be delivered in a single pulse that is fast enough and has small enough Coulombic broadening of the beam and of its energy distribution—so-called space-charge effects—the measurements might at least outrun the permanent structural disordering of the specimen that occurs as a result of massive radiolysis: Much of the damage that occurs as a result of "high" electron exposures, such as bending or shifting of the object, might never be observed if the exposures could be made short relative to the evolution time of that damage.

The probability that these or any other schemes will make it possible to bypass some of the current limitations in biological electron microscopy might reasonably be estimated to be zero, based on what we know today. The significance of finding a way to mitigate the effects of radiation damage would, on the other hand, be infinite (to hyperbolize just for effect), and the product of the two is indeterminate. Prudence thus requires that I close with the caution—indeed the hope—that unknown technologies may yet be contrived, as Abbe put it, that will bypass some of the current limitations of radiation damage.

I thank Mark Sales for first pointing out to me the possibility of interaction-free imaging in cryo-EM.

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