Cryoelectron microscopy pioneers win chemistry Nobel

Proteins frozen in vitreous ice are now being imaged with atomic resolution.

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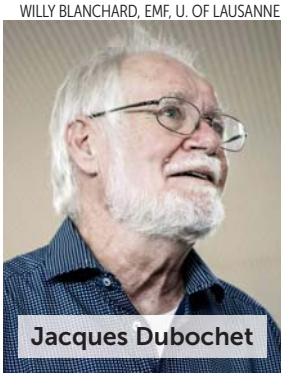

Jacques Dubochet

BRIAN WINKOWSKI, COLUMBIA U. MED. CTR.

Joachim Frank

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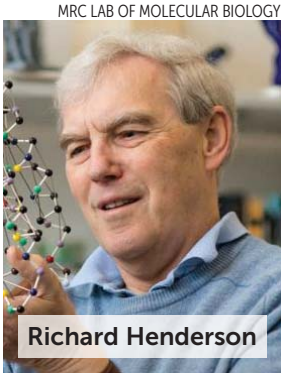

Richard Henderson

"This year's prize," announced Secretary General Göran Hansson of the Royal Swedish Academy of Sciences on 4 October, "is about a cool method for imaging the molecules of life." Indeed, though its samples are chilled to liquid-nitrogen temperature, single-particle cryoelectron microscopy (cryo-EM) faithfully captures the structures of biomolecular systems in their native aqueous environment. (See the article by Bob Glaeser, PHYSICS TODAY, January 2008, page 48.) As a result of recent and continuing technical developments, cryo-EM is poised to overtake x-ray crystallography as the technique of choice for solving the structures of large biomolecules—with the significant advantage that the molecules needn't be crystallized.

Three researchers who laid the foundations of modern cryo-EM will share the 2017 Nobel Prize in Chemistry. Jacques Dubochet, an emeritus professor at the University of Lausanne, devised the technique for embedding samples in thin films of vitreous, or amorphous, ice. Joachim Frank, now at the Columbia University Medical Center, recognized the need to average images of an ensemble of molecules, and he developed the first computational methods for doing so. Richard Henderson, of the MRC Laboratory of Molecular Biology in Cambridge, UK, obtained the first atomic structure of a protein by electron microscopy, and he spurred the field on by recognizing its potential.

Damage limitation

The challenge in using electrons to image biological specimens comes down to the competition between elastic scattering, which yields useful structural information, and inelastic scattering, which breaks bonds and damages the sample. Delicate biomolecules can tolerate only a small electron dose before being damaged beyond recognition.

An early workaround was so-called negative staining: drying the specimen in a solution of heavy-metal salt to form a thin salt cast around the structure of interest. The metal atoms—usually uranium—are far stronger elastic scatterers than carbon, nitrogen, and oxygen are.

In the late 1960s, when Frank was a graduate student, his mentor Walter Hoppe was working on a method for reconstructing the three-dimensional structure of a negatively stained molecule by imaging it from many angles. "The flaw with that approach," says Frank, "is that the process of imaging destroys the molecule—so whatever is reconstructed in the end is meaningless."

If the dose could be spread among many identical molecules, on the other hand, the structure could be solved without appreciably damaging any of them. Making and imaging the requisite number of identical molecules is easy. The hard part is to identify, sort, and align their noisy images to obtain a meaningful average. Frank's first insight was that it was even possible.

The drying process of negative staining often causes molecules to orient in a particular way with respect to the substrate. For example, in the micrograph in figure 1a of a subunit of the human ribosome, the seahorse-shaped molecules need only be sorted into two groups: those lying on their left side and those lying on their right. Frank and his colleagues developed techniques to seek out and align molecular images by calculating such a micrograph's auto- and cross-correlation functions and to sort them into clusters of similar images by using multivariate statistics.[1] Figure 1b shows part of the gallery of aligned images, and figure 1c shows the resulting average.

Figure 1c is just a 2D projection of a 3D molecule. Available methods could combine 2D projections into a 3D structure—as long as the relative orientation of each projection was known. For an ensemble of preferentially oriented molecules, Frank and his group realized that it sufficed to image the sample just once more, after tilting it by 50° or so with respect to the beam. Because the molecules are randomly positioned within the plane, the tilted image contains the full complement of 3D information.

When molecules lack a preferential orientation, Frank's correlation and cluster analyses still work to sort images into groups with almost the same 3D orientation. Determining that orientation is a much harder problem. The most common approach, developed later by others, is based on the so-called central section theorem, which states that the Fourier transform of each 2D projection is a slice through the center of the 3D Fourier transform of the object.

Glassy water

Negative staining, though quick and easy to implement, has its resolution fundamentally limited to around 15–20 Å by the granularity of the dried stain. And it requires taking biomolecules out of their aqueous environment, which can change their structure. Liquid-water samples can't survive the electron microscope's vacuum. Freezing samples into ordinary crystalline ice doesn't work either: The crystals damage the biomolecules and distort the image.

Fortunately, water has a glassy phase, metastable at temperatures below 135 K, with a structure similar to the liquid. Vitreous ice had been produced in the lab since 1935 by condensing water vapor onto a cold metal substrate, and the idea

of using it in electron microscopy dates to the 1940s. But a biomolecular solution must be vitrified directly from liquid; to prevent regular ice crystals from forming, the temperature would have to drop from 273 K to 135 K in a fraction of a millisecond. A generation of researchers tried and failed, and many concluded that the task was impossible.

Dubochet, by his account, was ill informed of the difficulty of vitrifying water when he started working on the problem with Alasdair McDowall. So, it seems, were Peter Brüggeller and Erwin Mayer of the University of Innsbruck in Austria. Both teams succeeded in the early 1980s with a similar combination of tactics to achieve quicker cooling: reducing the sample to micron-scale dimensions and using a secondary cryogen—liquid ethane or propane—cooled with liquid nitrogen.[2]

Within a few years, Dubochet had refined the technique into the one that's used, with little modification, in cryo-EM today.[3] A small drop—a few microliters—of the biomolecular solution is placed on a carbon-coated grid some 3 mm in diameter. Most of the liquid is blotted away with filter paper, leaving behind a microscopically thin film spanning the grid's micron-scale holes. Plunging the grid into liquid ethane or propane vitrifies the solution.

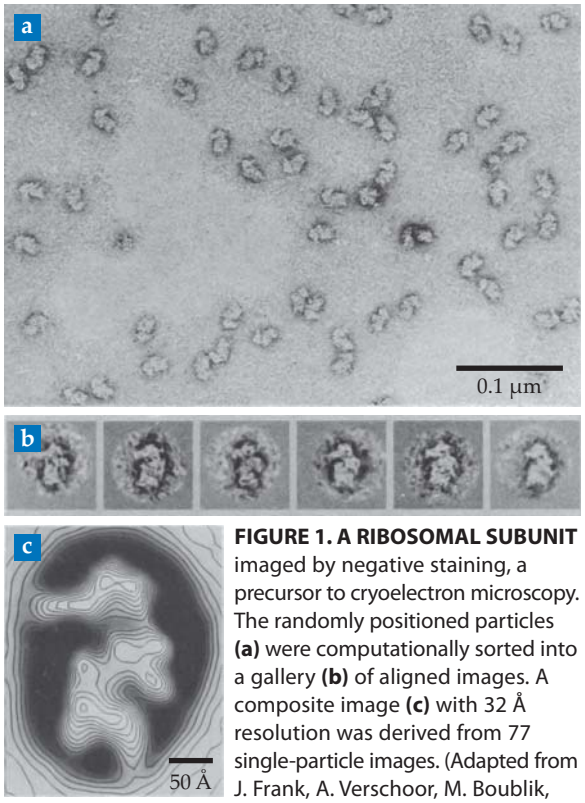

FIGURE 1. A RIBOSOMAL SUBUNIT imaged by negative staining, a precursor to cryoelectron microscopy. The randomly positioned particles **(a)** were computationally sorted into a gallery **(b)** of aligned images. A composite image **(c)** with 32 Å resolution was derived from 77 single-particle images. (Adapted from J. Frank, A. Verschoor, M. Boublik, *Science* **214**, 1353, 1981.)

Purple membrane

Henderson's first work on electron microscopy of biomolecules preceded both Frank's and Dubochet's. In 1975 he was interested in the protein bacteriorhodopsin, which pumps protons across the cell membranes of certain bacteria (see PHYSICS TODAY, May 2017, page 16). It naturally congregates in micron-sized patches of so-called purple membrane, and importantly, the molecules in each patch form a regular array—a natural 2D crystal. An electron beam transmitted through the membrane is diffracted into a pattern of spots, similar to those in x-ray crystallography.

Henderson and his colleague Nigel Unwin protected the membrane patches by drying them in a glucose solution; the procedure was similar to negative staining, except that the electrons were scattered predominantly by the protein, not the stain. From diffraction patterns collected at different beam angles, they derived a 3D structure with a resolution of 7 Å in the plane of the membrane and 14 Å perpendicular to it.[4] Their results showed that each protein molecule consists of seven closely packed rod-like structures, which they correctly interpreted as α helices spanning the thickness of the membrane.

By 1990, resolution had improved through developments in sample preparation, the microscopes themselves, and diffraction-pattern analysis. In particular, Henderson and colleagues had come up with a way to piece together purple membrane patches to make protein crystals up to 5 μm across. Although the out-of-plane structural resolution was still poor, at 10 Å, the in-plane resolution was 3.5 Å, good enough to start fitting an atomic model.[5]

Chemical bonds in biomolecules are 1–1.5 Å long, so individual atoms were far from discernable. But the atomic model didn't need to be built from scratch. Many amino acids have characteristic shapes that can be identified in a 3.5 Å map. If a protein's sequence is known—as bacteriorhodopsin's was—then nearby amino acids can also be slotted into the structure. The established rules of how the components of folded proteins position themselves, gleaned from decades of x-ray structures, allow the conformation to be refined even more precisely.

Blob-ology

Similar results were slow in coming for other proteins. Bacteriorhodopsin is almost unique in forming natural 2D crystals; more often, making a 2D crystal is even harder than making a 3D crystal for x-ray crystallography. There are a few exceptions. The cytoskeletal protein tubulin, for example, normally assembles into tubes, but it can be made to form ordered sheets. It was one of the next proteins to have its atomic structure solved by electron microscopy (see PHYSICS TODAY, March 1999, page 21).

On the other hand, single-particle cryo-EM—experiments using Frank's algorithms and their successors to pick out and average images of many unconnected molecules—yielded little more than poorly resolved blobs. Still, the field attracted a small but devoted following of researchers interested in molecules too large or too floppy to crystallize. "We didn't care that we were getting blobs," says Eva Nogales of the University of California, Berkeley, "because getting blobs was better than getting nothing."

In 1995, prompted by a conversation on the future of funding for biomolecular imaging, Henderson published a detailed review comparing the fundamental limits of microscopy with electrons, neutrons, and x rays.[6] He argued that, per useful elastic scattering event, electrons inflicted by far the least damage on the sample. And he calculated that under perfect imaging conditions—detection of all elastically scattered electrons and no noise sources other than counting statistics—single-particle cryo-EM images could be aligned well enough to produce structures of resolution 3 Å or better. Only images of the smallest proteins, of mass less than 38 kilodaltons, contain too little information to be aligned.

Of course, conditions were nowhere close to perfect. The detectors of the

day—photographic film and CCD cameras—captured at most 30% of the electrons, and images were blurred by beam-induced distortion of the ice films. But Henderson's analysis changed the perception of what was possible, and the improvements came. Microscopes' electron beams got brighter, their stages steadier, and their vacuums better. New software packages explored different approaches for classifying and combining images. By 2008, virus structures were nearing 3 Å resolution—the viruses' large size and high symmetry made them among the easiest structures to align and analyze.

New horizons

The revolution came a few years later with the advent of direct electron detectors. Both efficient and fast, the new detectors not only capture 50–80% of incident electrons but also allow beam-induced image blurring to be computationally undone. "Before the new detectors, nobody was getting atomic resolution," says Nikolaus Grigorieff of the Janelia Research Campus in Ashburn, Virginia. "With the new detectors, everybody was." Although individual molecules still present experimental challenges, resolutions between 2.5 Å and 3.5 Å are now routine.

One of the most exciting potential applications of the new high-resolution structures is drug development. (See PHYSICS TODAY, August 2016, page 13.) Typical modern pharmaceuticals are relatively small synthetic molecules that target a specific site on a much larger protein, perhaps to block or mimic the effect of some other molecule. Identifying the best molecules for the job is aided considerably by good 3D structures of the target protein and the protein–drug complex.

Since the early 1990s, pharmaceutical companies have used x-ray structures to help in drug design. But many drugs target hard-to-crystallize membrane proteins. Cryo-EM can help, especially if its resolution can be pushed further. An image at 3.5 Å resolution gives atomic structure only in conjunction with a general knowledge of what folded protein structures look like, and pharmaceutical interlopers can reorganize a protein in subtle, unknown ways. Ideally, then, the resolution should be good enough to observe amino-acid orientations directly, as exemplified in figure 2.

Better resolution may come through advancements in sample preparation. For all the developments in other parts of the cryo-EM system, Dubochet's technique for creating thin films of vitreous ice has hardly changed in 35 years. And there's room for improvement. Blotting the excess fluid with filter paper means that most of the sample ends up in the trash—a waste of valuable protein.

Moreover, blotting offers little control over the ice film's thickness. When the ice is thicker than two to three times the molecular dimensions, the resulting images are noisy. Too thin a film can also be a problem: Before freezing, molecules are trapped between two air–water interfaces, which can cause proteins to unravel. "There are tricks you can use to stabilize the molecules," says Nogales, "but you have to take it case by case, and sometimes nothing works."

Bridget Carragher and her colleagues at the New York Structural Biology Center are among the groups working on the sample preparation problem. Their automated system, called Spotiton, uses a piezoelectric dispenser to spit picoliters of solution directly onto a grid just as it enters the liquid ethane. The grid is "self blotting," with nanowire-covered surfaces to wick away excess fluid.

Over the past few years, scientists of all types have flocked to cryo-EM. They include structural biologists, of course, but also computer scientists, physicists, and data scientists, all bringing new perspectives and ideas to the technique. Cutting-edge developments include the use of novel phase plates to increase contrast between the scattered and unscattered electrons (see PHYSICS TODAY, September 2017, page 22); search methods for detecting individual protein molecules, whose isolated structures are known to high resolution, in low-resolution micrographs of whole cells;[7] and new ways of analyzing cryo-EM snapshots of floppy molecules to derive not static structures but continuous paths of conformational change.[8]

"But none of this gets done without funding," says Carragher. Cryo-EM labs—from the electron microscopes and computers to the expert staff who operate them—are expensive to set up and maintain, and not every institution can afford its own. Across Europe and Asia, shared user facilities have been springing up to allow more equal access to cryo-EM, much like synchrotron user facilities do for x-ray crystallography. The National Institutes of Health opened a national cryo-EM facility in Frederick, Maryland, in May 2017 and is reviewing proposals for more US facilities. Once they're built, Carragher says, "the field is going to the stratosphere, as far as I can tell."

Johanna Miller

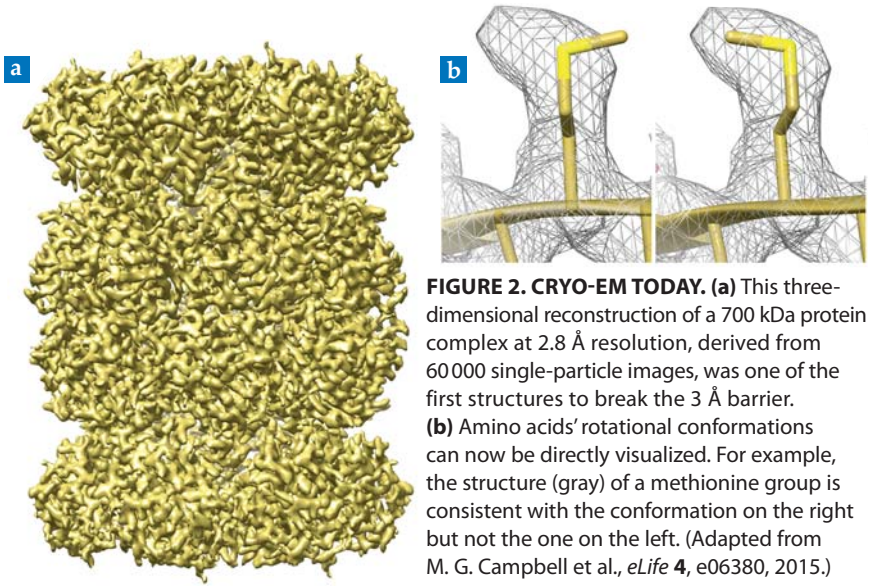

FIGURE 2. CRYO-EM TODAY. (a) This three-dimensional reconstruction of a 700 kDa protein complex at 2.8 Å resolution, derived from 60 000 single-particle images, was one of the first structures to break the 3 Å barrier. **(b)** Amino acids' rotational conformations can now be directly visualized. For example, the structure (gray) of a methionine group is consistent with the conformation on the right but not the one on the left. (Adapted from M. G. Campbell et al., *eLife* **4**, e06380, 2015.)

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