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How Cryo-EM Became so Hot

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Abstract

The Royal Swedish Academy of Sciences awarded the 2017 Nobel Prize for Chemistry to Jacques Dubochet, Joachim Frank, and Richard Henderson for “developing cryoelectron microscopy for the high-resolution structure determination of biomolecules in solution.” Achieving this goal, which required innovation, persistence, and uncommon physical insight, has broadened horizons for structural studies in molecular and cell biology.

Cryo-electron microscopy—or single particle cryo-EM, as the method has come to be known — brings a number of desirable, new features to structural biology that were not previously available with other methods of high-resolution structure determination. The technique allows macromolecules to be studied in more “native,” i.e., biochemically functional buffer conditions, as opposed to only the particular conditions in which the molecules may happen to crystallize. The observed conformations are likely to be functionally relevant, as off-pathway conformations will not be selected for by crystal packing forces. In addition, it is possible to determine structures for macromolecules in two or more functional states that are in equilibrium with one another. Lastly, the method relies on only a few microliters of sample, which can be at concentrations as low as tens of nanomolar. Taken together, it is now possible to solve structures for macromolecules that previously had proven to be too difficult to crystalize or too large to study with NMR. A few examples include atomic-resolution structures of ribosomes in solution (Bai et al., 2013), γ-secretase (Bai et al., 2015), and ion channels (Liao et al., 2013). Structures have been obtained at resolutions as high as 2.2 Å (Merk et al., 2016) and of proteins as small as the 64 kDa hemoglobin molecule (Khoshouei et al., 2017).

Using an electron microscope to see biological macromolecules in atomic detail seemed an obviously good idea when it was first proposed. As Richard Feynman famously asserted in a lecture to the American Physical Society in 1959 (Feynman, 1960), when referring to the use of the electron microscope, “It is very easy to answer many…fundamental biological questions; you just look at the thing!” Physicists knew, some 30 years before that, that high-

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energy electrons are waves, whose wavelength is much less than interatomic distances. They also knew that these waves can be focused by magnetic lenses. As a result, they realized that the diffraction limit to image resolution would not be a problem for electron microscopy, as it is for light microscopy. Furthermore, images can be much more powerful than diffraction patterns. Although the two are mathematically related to each other by the operation of Fourier transformation, information that is crucial for structure determination, called “the structure-factor phases,” can be recovered directly from the image, whereas it is more difficult to recover this information from diffraction patterns. It eventually became apparent, how-ever—especially by the early 1970s—that seeing biological macromolecules in atomic detail would not be as simple as first thought. Instead, major innovations would be required in order to make this happen.

First of all, electron microscopes have to be operated at high vacuum in order to avoid unwanted scattering of electrons by anything other than the specimen. However, native protein structure would be only poorly retained, if at all, unless specimens could somehow remain well hydrated while in the vacuum of an electron microscope. Achieving this seemed to be asking for the impossible, until Taylor and Glaeser (1974) showed that high-resolution structure is retained when previously frozen specimens are kept in the microscope at low temperature. This approach did not gain widespread use, however, until Jacques Dubochet and his colleagues discovered that thin samples could be frozen rapidly enough to keep the water molecules in a non-crystalline structural arrangement similar to that of liquid water (Dubochet et al., 1988) (Figure 1). The basic method developed by Dubochet continues to be used today.

In addition, the attempt to see biological macromolecules in atomic detail had to face the fact that high-energy electrons are not just short-wavelength waves, but they are also ionizing radiation. As a result, radiation sensitivity forces the experimentalist to limit the electron exposure of the samples so severely that it results in very noisy images. As a consequence, data have to be merged from many thousands of structurally identical molecules in order to reduce the noise while at the same time ensuring that the radiation dose received by any one of them does not destroy the object. The problem of doing this in practice was solved by Richard Henderson and Nigel Unwin by using images of two-dimensional (2D) crystals (Figure 2A), for which merging the image data to obtain a three-dimensional (3D) structure is highly practical (Henderson and Unwin, 1975). Even so, collecting data from tilted 2D crystals, which is required to obtain 3D structures, remained very problematic. Furthermore, obtaining well-ordered 2D crystals proved perhaps even more problematic, and the technique gradually lost popularity.

At about the same time that work with 2D crystals was just beginning, Joachim Frank (Frank, 1975) first proposed — and, subsequently, Saxton and Frank (1977) showed — that merging data from images of single molecules could, in theory, also work for non-crystalline samples (Figure 2B). Frank’s experimental pursuit of this approach began with ribosomes and other very large macromolecular assemblies, initially using negatively stained samples but subsequently moving on to using unstained “cryo” specimens. Although steady improvement was made and others even achieved atomic resolution for icosahedral virus particles and other structures with high symmetry, the results with asymmetric or low-

symmetry particles seemed to stall at a point short of what is needed to trace the peptide chain. In retrospect, it is evident that there still were more technical problems that had to be overcome.

One such problem, suspected for many years, turned out to be the fact that cryo-EM specimens move when they are irradiated. A solution to this problem was demonstrated by Grigorieff et al., making use of a greatly improved camera with fast readout speed and improved signal-to-noise ratio to record images as a series of frames, each taken with a small fraction of the total allowed electron exposure. This camera made it possible to align frames before summing them, thereby eliminating the motion that occurred from one frame to the next (Campbell et al., 2012). Work to develop new cameras of this type, based on direct detection of electrons rather than their conversion to light with a scintillator, was first pioneered for applications in cryo-EM by Henderson et al. (see, for example, Faruqi et al. [2005]). A version that is capable of counting each electron (Li et al., 2013) is currently the preferred type of camera in the field.

The resulting, improved cryo-EM images became available at almost the same time that new image analysis approaches emerged. The most significant step involved use of a Bayesian approach (Scheres, 2012). The new software tools provide a powerful way to take into account possible heterogeneity in composition and in conformation, resulting in more robust reconstructions (Bai et al., 2013).

Looking back over the advances summarized above and seeing the many high-resolution structures obtained by single-particle cryo-EM in the past 5 years, it once again seems a simple thing to imagine what might be possible next. At the level of isolated single particles, work has begun to obtain experimental images that show the conformational changes made by molecular machines at various steps in their biochemical cycles and to estimate the thermodynamic landscape along their reaction pathways (Dashti et al., 2014). At the level of cell biology, cryo-EM has also been used to obtain tomographic reconstructions of the thin margins of cultured cells, and more recently, this has been extended to thin lamellae cut out from frozen cells with a focused ion beam. One is thus tempted to envision, along with Leis et al. (2009), that cryo-tomogram s will serve as “3D representations of the entire proteome and…[as] snapshots of the interaction networks underlying cellular functions.”

Only a small part of this futuristic program is achievable today, and it seems that little more would be possible in these directions using only existing methods and technologies. Fortunately, however, there is a lot of opportunity for further improvement. As an example, use of the single-particle method can be dramatically hampered by shortcomings of the present cryo-EM sample-preparation techniques. One cause may be the destructive nature of the air-water interface to which particles are exposed in the very thin samples that must be created just before vitrification. In addition, the performance of direct-detection cameras can be enhanced considerably, and improved phase-contrast devices for electron microscopes may be on the horizon. Together, these two advances could make it possible to more effectively mitigate the effects of beam-induced motion, a problem that currently remains severe when specimens are tilted to high angle. As work in this field progresses, it can be expected—as before—that new surprises and limitations will appear, which will call for a

new wave of innovation and creativity. Thus, while the 2017 Nobel Prize in Chemistry celebrates the past steps in the development of cryo-EM that opened up new capabilities in structural biology, this is far from being the final word.

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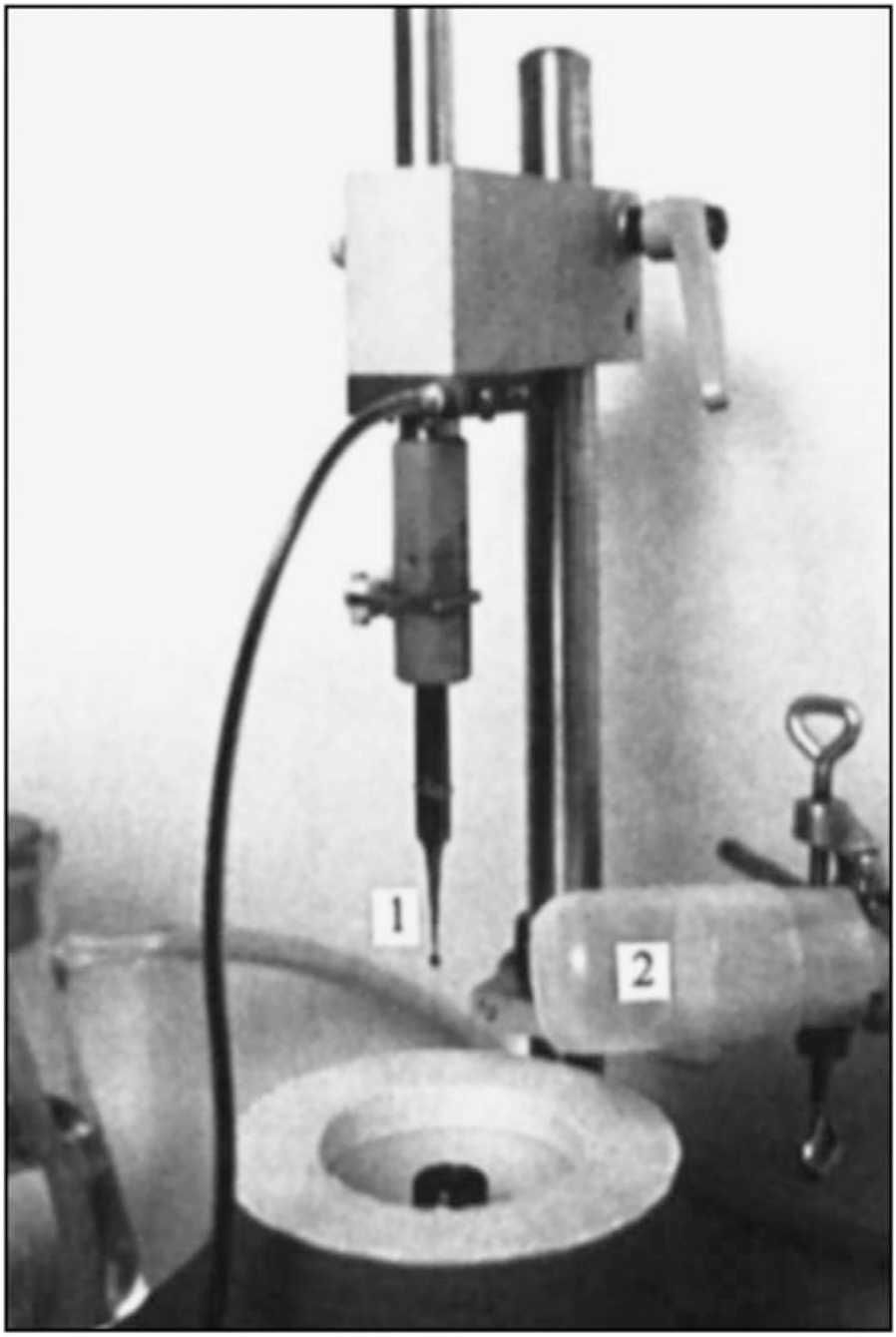

Figure 1. Rapid Freezing Results in Vitreous Ice

Biological macromolecules must be surrounded by water if they are to retain their native structure and function. In order to prevent that water from evaporating when a fully hydrated sample is inserted into the vacuum of an electron microscope, Dubochet et al. (1988) used the elegant but simple apparatus shown here to freeze samples. In such frozen samples, water molecules remain in a nearly random arrangement, as they are in the liquid, and thus, the biological macromolecules remain in a near-native state.

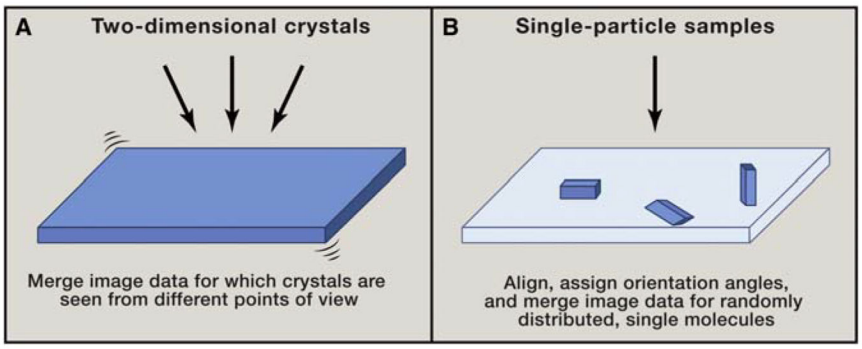

Figure 2. Crystals Consist of Identical Objects with a Known Spatial and Angular Relationship

(A and B) A 2D protein crystal is made up o f a layer of molecules that, in this cartoon, all have the same orientation and w hose respective locations are specified by points on a regular lattice (A). Henderson and Unwin (1975) took advantage of 2D crystals to facilitate merging data from many thousands of bacteriorhodopsin molecules, thus building up sufficient statistical definition of the signal without exposing any one molecule to more radiation than it could tolerate. Such images had to then be recorded for different crystals, tilted by different amounts, in order to obtain the data needed for a 3D density map. Rather than relying on crystalline forms, however, single-particle cryo-EM uses an ensemble of randomly dispersed macromolecules (B). Saxton and Frank (1977) demonstrated that, in principle, information about the locations and orientations of these molecules could be obtained computationally from their images. In doing so, “virtual crystals” would effectively be grown *in silico*, in the sense that it is then possible to merge data from many thousands of identical molecules.