PROFILE

Profile of Joachim Frank, Richard Henderson, and Jacques Dubochet, 2017 Nobel Laureates in Chemistry

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In 2017, the Nobel Prize in Chemistry was awarded to three key pioneers in the birth and development of cryoelectron microscopy (cryo-EM): Jacques Dubochet, Joachim Frank, and Richard Henderson. As a starting graduate student in England, moving from physics to biology and the study of macromolecular structure, I had the fortune of attending one of the early conferences in the, at the time, embryonic field of cryo-EM. It was 1990, and Jacques Dubochet (Fig. 1), whom I met at the conference, and coworkers (1) had provided the scientific community a few years before with a simple, highly practical way to "vitrify" solutions of biological samples that they used, for example, to visualize intact viral particles (2). Dubochet's charming recounting of the early days in his own words can be found in a recent perspective (3). By then, there was already access to commercially available cryoholders to use standard transmission electron microscopes for the study of frozen-hydrated samples. Thus, major practical bottlenecks had already been overcome, but the cryo-EM community could still be counted on one's fingers. That year of 1990 saw the publication of the long-time-coming atomic model of bacteriorhodopsin using electron crystallography (not quite vitrified, discussed below) by Henderson et al. (4) (Fig. 2). This publication was the needed demonstration that electrons could provide this kind of detailed structural description of radiation-sensitive biological samples. That paper became a true inspiration for me and for a whole generation of cryo-EM practitioners.

While there was a very long history of the electron microscope's use to visualize both cellular ultrastructure and molecular architecture, few were aware, at the time, of the capabilities of the technique to provide the type of resolution that, until then, only X-ray crystallography had offered. The major limitations were radiation damage, preservation of biological structure in the high vacuum of the scope, and the low contrast of the sample made of light atoms. The application of transmission electron microscopy (TEM) to inorganic samples that do not suffer from such limitations had already rendered near-atomic-resolution images at the time; now, with aberration corrected scopes, such inorganic materials can be visualized at better than 1-Å resolution.

Fig. 1. Jacques Dubochet. Image courtesy of Félix Imhof (photographer), © Université de Lausanne (UNIL).

One of the first proofs that high-resolution EM data were attainable for protein samples came in 1975 from Bob Glaeser, whom I also first met at the 1990 conference in England, and who would, a few years later, become my colleague and friend at the University of California, Berkeley. Glaeser and his student, Ken Taylor, demonstrated that frozen-hydrated 2D crystals of catalase bombarded with high-energy electrons could render atomic resolution under cryogenic conditions if the electron dose was limited (5). This seminal work became the driving force for Dubochet's efforts to vitrify biological samples, as he recounts himself (3). Still, for many years,

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Fig. 2. Richard Henderson. Image courtesy of Medical Research Council (MRC) Laboratory of Molecular Biology.

most studies of biomolecules were carried out using negative stain, a technique in which what is visualized is a dried cast of the sample made of a heavy atom salt, typically uranium-based. The negative stain overcomes the bottlenecks mentioned above, as the contrast is high; the vacuum is not a problem for the dried sample; and the inorganic salt is not as radiation-sensitive as the macromolecule itself. However, negative-stain studies are, by default, limited in resolution by the grain size of the uranyl salt, and certain samples simply collapse when dried out. For a while, the closest approach to a hydrated sample was to embed it in a low concentration of a sugar solution, such as glucose, trehalose, or tannic acid. Indeed, these media were used in electron crystallographic studies to produce the atomic models of bacteriorhodopsin first, and then of the plant light-harvesting complex and tubulin by Henderson et al. (4), Kühlbrandt et al. (6), and Downing and coworkers (7) during the 1990s, the glory days of electron crystallography. While this technique seemed to be the method of choice at the time for high-resolution cryo-EM studies, technical difficulties having to do with the requirements to tilt the sample and the lack of flatness in the crystals made the process inefficient and limited to a very small percentage of the few successful 2D crystals obtained out of many attempts. Despite the dramatic effect of the 1.8-Å structure of aquaporin, published by Walz and coworkers in 2005 (8), it was obvious from the very low throughput of electron crystallography that this methodology could never keep pace with its X-ray counterpart.

Fig. 3. Joachim Frank. Photograph by Brian Winkowski and image courtesy of Columbia University Medical Center.

Fourier–Bessel methods, first used in the pioneering work of De Rosier and Klug (9), were being used for helical protein arrangements, both natural and induced. Among my own personal favorites were the study of frozen-hydrated helical arrays of the acetyl choline receptor by Unwin (10) and the naturally helical microtubules studied by Wade and coworkers (11), Amos and coworkers (12), and Milligan and coworkers (13). Common lines principles, on the other hand, were also robustly producing the structure of viruses. Both approaches, whether using icosahedral or helical principles, were first used for negatively stained samples but benefited tremendously from the advent of sample vitrification and cryo-EM methodologies. A resolution landmark came with the structures of the hepatitis B virus capsid at subnanometer resolution by Crowther and coworkers (14) and Steven and coworkers (15). Thus, at the turn of the millennium, biological assemblies with a high degree of symmetry appeared for most to be the only type of samples for which structural characterization by TEM could reach any significant resolution. This limitation would certainly had kept cryo-EM as a niche technique, gathering the attention of few, with successes in obtaining atomic resolution that appeared more the exception than the rule.

It was in the context of a high-symmetry-dominated field that Joachim Frank (Fig. 3) and his colleagues had the vision and the mathematical resourcefulness to pursue the study of low- or no-symmetry objects. All other methods of EM 3D structure determination used Fourier inversion, just like X-ray crystallography, as a principle for reconstruction. The discrete nature of the Fourier transform of a 2D crystal or a helix allowed the researchers to use filtration methods to increase the signal-to-noise ratio in the otherwise very noisy images of biological samples that result from the short electron exposure used to limit radiation damage. Although not as sparse, the highly redundant Fourier transform of a virus, due to its 60-fold symmetry, provided a significant advantage to the image processing. Frank and his colleagues envisioned a methodology, relying more on real, rather than reciprocal, space to align experimental, noisy images of a biological object with low or no symmetry, to build the signal by averaging. Initially using negatively stained samples, Frank et al. (16) were able to produce first 2D class averages, and, soon after, van Heel and Frank (17) were able to use multivariate statistical analysis to classify different

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views of the object. A number of approaches were then developed for 3D reconstruction relying either on geometrical principles, like the random conical tilt method of Radermacher and Frank (18), common lines in a real-space implementation (angular reconstitution) of van Heel (19), or reference-based approaches as implemented by Penczek and Frank (20). The single-particle 3D reconstruction field was taking shape, and Frank et al. (21) used the ribosome as a driver, test sample for the implementation of this evolving methodology.

Because of the noise in cryo-EM images, the "computational" alignment was not as accurate for a long time as the "chemical" alignment provided by 2D crystals, helical samples, or icosahedral viruses, and the resolution of the initial single-particle reconstructions was limited. I still remember when claims of better than 25 Å resolution for a ribosome reconstruction were being questioned as practically unfeasible in the 1990s at an international conference. However, the conferences showed higher and higher resolution each year, single-particle methods became more and more broadly used, and the advantage of their general applicability made single-particle cryo-EM the method of choice to study samples that resisted crystallization, even when the 3D reconstructions were limited to "blobs" of 30- to 15-Å resolution. Within this "blobology" regime, the use of hybrid approaches that combined the low-resolution cryo-EM reconstruction of a macromolecular assembly with the atomic crystallographic structure of protein components or domains proved extremely helpful in a large number of studies for our understanding of the architecture of large complexes.

Would cryo-EM ever be sufficient to directly and generally provide atomic-resolution structures? A critical landmark paper was that of Henderson (22) proposing, already in 1995, that, based on physics principles, cryo-EM images should provide 3-Å resolution structures with just 10,000 images of the object for macromolecules as small as 100 kDa, without any assumption of symmetry. Soon after, Glaeser (23) proposed an even more optimistic estimate. Because the calculations stood very far from what was possible at the time, it was clear that some experimental bottlenecks were holding the field back. Among them was the poor performance of electron detection media that further deteriorated the poor signal-to-noise ratio of the images. Traditionally, recording was done on photographic film, but CCD cameras, with a scintillator that converted the high-energy electrons into photons, became popular and allowed the development of automatic data acquisition practices, such as those pioneered by Potter et al. (24). Unfortunately, the performance of CCDs was even worse than that of traditional film. Another major bottleneck was the recognized, although poorly understood, problem of beam-induced motion that resulted in blurring of a majority of cryo-EM images. A dramatic improvement came about with the advent of new direct electron detector technology, which ultimately led to what has come to be known as the "resolution revolution" in cryo-EM. These detectors, which implemented different engineering solutions to solve the problem of detector damage by the high-energy electrons (direct electron detectors have been reviewed recently in ref. 25), ultimately provided higher contrast and very fast readout. These properties were soon utilized by Grigorieff and coworkers (26) to implement a movie-mode of data collection that could then be used for the alignment of frames and the correction of a significant part of the beam-induced motion. Thus, the new detectors resulted in images that had both higher contrast and higher resolution. In turn, these better images started to be analyzed with greatly improved and friendlier software packages, among them the Bayesian-based approach of Scheres and coworkers that delivered atomic structures of the ribosome with as little as 30,000 ribosome images (27). This and others' advanced and easy-to-use data analysis packages have become capable of identifying and characterizing multiple states, both compositional and conformational, that coexist in the test tube and the cryo-EM images, thus giving a new functional dimension to structure determination (28–30). Large, unstable complexes, available in minute amounts, have now been obtained in different functional states (31). An ever-increasing number of integral membrane proteins, the "bête noire" for crystallographic analysis, are now being obtained by cryo-EM (32). Importantly, the theoretical limits defined by Henderson (22) and Glaeser (23) are coming closer: The resolution attainable for the best-behaving samples have now allowed the visualization of ordered water molecules (33), and near atomic resolution has now been obtained for molecules as small as 60 kDa using phase plate technology (34).

From the trickle of atomic structures produced by electron crystallography around the 1990s, to what seems like a flood of structures during the last four to five years, including for some very challenging biological samples, the cryo-EM field has come a long way, and now deserves its rightful place as a mainstream technique for structure determination. We all owe a big debt of gratitude to Dubochet, Frank, Henderson and the rest of the cryo-EM pioneers who had the vision and the capability to break down technical barriers, opening the door to discovery that so many are going through today. Major improvements still await, and our shiny Nobel Prize winners are an inspiration for present and future work to take cryo-EM to the next level.

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