



Scientific Background on the Nobel Prize in Chemistry 2017

## THE DEVELOPMENT OF CRYO-ELECTRON MICROSCOPY



The Royal Swedish Academy of Sciences has decided to award **Jacques Dubochet, Joachim Frank** and **Richard Henderson** the Nobel Prize for Chemistry 2017 for "developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution".

## Introduction

In 1968, George Gamow, a physicist, and Martynas Yčas, a microbiologist, published a popular-science book, *Mr. Tompkins Inside Himself: Adventures in the New Biology*, which tells a story about Mr. Tompkins as he explores the cellular architecture of his body on a dream journey through his bloodstream, guided by his doctor. While inspecting the structural details of single cells and organelles, Mr. Tompkins' guide enthusiastically informs him that this knowledge is based on studies using the electron microscope.

Mr. Tompkins' tour makes it obvious that at that time the instrument had already brought studies of biological material to a previously unimaginable level of detail. However, until just a few years ago scientists could still only dream of being able to use the electron microscope to zoom in further into cells and organelles, in order to uncover the atomic details of the biomolecules that underpin their architecture and function.

This dream became reality recently when a series of critical developments made it possible to take full advantage of the pioneering discoveries and improvements made by Jacques Dubochet, Joachim Frank and Richard Henderson. These advances now allow structural determination of non-crystalline biomolecules in solution at high resolution, using single-particle<sup>1</sup> cryo-electron microscopy (EM).

## Challenges in structural studies of biological material

Short after the experimental demonstration of an **electron microscope** by Ernst Ruska, for which he was honoured with the Nobel Prize for Physics in 1936 (1), Ladislaus Marton published a paper (2) that commented on Ruska's discovery. In this short report, Marton noted that the new instrument unfortunately could not be used to study biological material without the "*destruction of the organic cells by the intense electronic bombardment*".

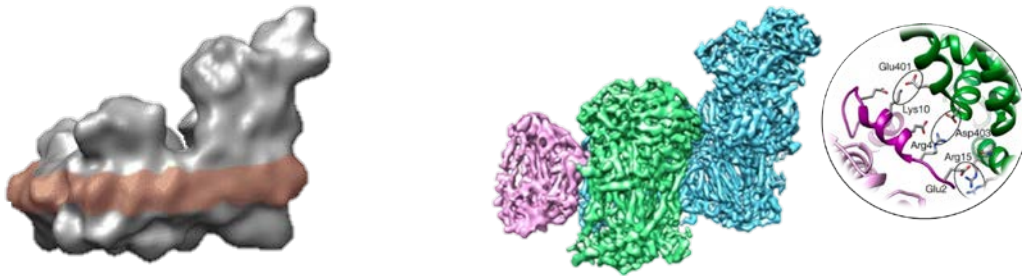
Preventing such destruction would require a new sample-preparation technique. Marton proposed visionary solutions to the problem: **cooling the biological material** or the use of an approach similar to negative staining. Another major problem was how to **preserve water** in the biological sample in the vacuum maintained inside the electron microscope chamber.

And there were even more challenges to face. To mention only the most basic ones, intact biological material has very **low** image **contrast** as most high-energy electrons pass straight through the specimen. At the same time, the **electron** dose must be kept **low** enough to **prevent damage**. The probability for multiple electron scattering events must be negligible at the

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<sup>1</sup> The term "particle" is used for biological macromolecules or complexes. The term "single particle" is used to indicate that non-crystalline specimens are analyzed. When using this approach, an ensemble of a large number of individual particles in solution is analyzed.

electron energy used; i.e. samples must be **thin**, ideally comprising a single layer of the particles of interest. Furthermore, the studied objects often move both upon interacting with electrons and due to drifts in temperature; the movement reduces information content, especially when using film or slow detectors to record images. As a result, until recently the **resolution** was typically limited to a few nanometres for biological molecules (**Fig. 1**).



**Fig. 1.** Models of the electron-transport chain components in a mitochondrial supercomplex  $I_1III_2IV_1$ , determined in 2011 (left, from (3), resolution  $\sim 2$  nm) and 2016 (right, from (4), resolution  $\sim 6$  Å), respectively. On the left, the coloured shape indicates the position of amphipols used for solubilization. On the right, complexes I, III and IV are shown in blue, green and pink, respectively. The encircled inset shows a model of a putative CIII-CIV interface.

## Negatively stained biological material

The necessity to use the **lowest possible electron intensities to study low-contrast** samples stimulated the development of new sample-preparation methods when recording images of biological material. The first commonly and successfully employed method was **negative staining**, established in the 1940s and refined during the following 20 years (5-7).

When using this approach, the biological material is embedded in a **thin amorphous film** of a **heavy-metal salt**, which generates a cast around the object. The cast scatters electrons more strongly than the encapsulated material, is more resistant to electron damage, and prevents collapse of the biological material during drying in the vacuum within the electron microscope.

The approach offered detailed information about the morphology of **bacteria, viruses and organelles**. However, for studies of **single molecules** or **molecular complexes**, in the best case the pictures could reveal only the envelope of the covered particles with a resolution that is limited by the granularity of the stain. Nevertheless, the use of this sample-preparation technique offered important low-resolution structural information. The experimental and theoretical tools used for **calculation** of **three-dimensional (3D)** structures from **two-dimensional (2D)** projections in the electron microscope established the basis for today's advancements.

## Negatively stained single particles

Aaron Klug (Nobel Prize for Chemistry in 1982) noted that verification of a 3D structure of a negatively stained particle from analysis of an electron micrograph requires observations from **different directions**. Such an analysis could be achieved either by tilting the specimen or by analysis of many particles positioned at different orientations (8). In the early 1960s, Klug and his colleagues designed optical methods for analysis of electron micrographs of periodic structures (9,10) and established methods to obtain structural information from electron diffraction of thin catalase crystals in the electron microscope (11).

Important contributions were also made around the same time by Walter Hoppe, who established new methods in electron crystallography to analyse protein structures (12). Hoppe also analysed non-crystalline macromolecules without symmetry, but the experimental approach used in these studies could only yield low resolution data (13) and the approach could not be generalized because of too-high accumulated electron doses that could be potentially damaging.

In 1968 David DeRosier and Klug (14) presented the first successful calculation of a 3D structural model from analysis of 2D projections in an electron microscope. The authors analysed the tail of the bacteriophage T4, which was chosen because of its helical symmetry. The 3D model could be calculated from an analysis of a single projection because the information content was equivalent to that obtained from projections of 21 different orientations of each subunit (14).

Calculation of 3D models of non-helical particles requires combination of data from several 2D projections of these particles in the electron microscope. One approach used to determine the relative orientation of particles that generate specific 2D projections was presented in 1970 by Anthony Crowther, together with DeRosier and Klug. The method, called the common lines approach, was used to determine a 3D envelope of icosahedral viruses based on an analysis of 2D projections in the electron microscope (15,16). The authors suggested that the method could also be applied to particles with no symmetry by tilting the sample, but it proved to perform best for symmetrical particles, such as the icosahedral viruses.

## Native protein crystals at room temperature

Techniques were also needed to preserve intact biomolecules in the **hydrated** state in the electron microscope and to determine conditions for non-destructive irradiation. Donald Parsons developed environmental chambers in which a humid atmosphere was maintained at room temperature in the electron microscope (17,18). Using this approach, he demonstrated electron diffraction from catalase crystals and showed that it is possible to find conditions under which the protein structure remains intact during electron irradiation.

Robert Glaeser quantified electron-induced radiation damage in studies of crystalline catalase and small organic molecules (19). He realized in the early 1970s that for unstained specimens,

care must be exercised to maintain **low electron doses** in order to minimize damage due to inelastic electron scattering. Glaeser also noted that using low electron doses requires **averaging** over ensembles of particles to increase the **signal-to-noise ratio**. In other words, the radiation would be distributed over multiple copies of the same particle (19-21).

Important progress was made in the mid-1970s by Henderson and Nigel Unwin, who developed a new preparation method that enabled studies of **unstained** protein crystals in the electron microscope at room temperature. Henderson and Unwin **replaced water** with a **glucose solution** to **preserve samples in vacuum** and used the new preparation method in studies of intact protein crystals. Radiation damage in the electron microscope was analysed systematically and the **electron intensity** was adjusted to a low level of  $\sim 1 \text{ e}^-/\text{\AA}^2$  to minimize the effects of electron radiation.

In an initial study, the authors presented projection maps of thin catalase crystals and 2D crystals of bacteriorhodopsin in a purple membrane (22). Images of the structures and of the diffraction patterns were obtained. The phases were calculated by Fourier transformation of the structure images. In a second study, the same authors tilted the specimen to collect projection images from different directions. Using these data they obtained a 3D map of bacteriorhodopsin, which revealed the general architecture of the protein, but still at a rather low resolution (23).

Analyses of the electron micrographs and calculation of the 3D map from the 2D projections were based on methods developed by Klug and colleagues (14,24), combined with the use of information obtained from the electron diffraction patterns. The very low contrast of  $\sim 1 \%$  limited the new preparation method to diffraction studies of crystals.

### **Native protein crystals at cryogenic temperatures**

Cooling the specimen was expected to reduce water evaporation and to protect the biological material from radiation-induced damage. Starting in the 1950s, Humberto Fernández-Morán explored the possibilities for freezing samples and preparing thin cryo-sections for studies using cryo-EM (25). However, upon freezing, water typically nucleates to form crystalline ice, which strongly diffracts electrons, thereby effectively obliterating signals originating from the sample. Furthermore, formation of ice crystals may change the specimen structure.

According to Paul Schmidt's historical overview (26), Basile Luyet had already noted the problems associated with the formation of crystalline ice when cooling cells in the 1940s. Luyet realized that the solution to the problem could be to cool the biological material sufficiently rapidly to preserve water in a liquid amorphous state, referred to as vitrified water. Cooling techniques were also introduced early for studies of protein crystals in the field of X-ray crystallography, where the formation of crystalline ice was prevented by using sucrose or glycerol as cryo-protectants (27,28).

In the electron microscopy field, Kenneth Taylor and Glaeser (29,30) demonstrated electron diffraction patterns from unprotected frozen catalase crystals to higher than 3-Å resolution. They showed that hydration was maintained in the electron microscope at cryogenic temperatures and contrast was improved compared to when using glucose. The available technology at that time only allowed studies above approximately -120°C, which is above the transition temperature from amorphous to crystalline ice. However, the authors did not observe crystalline ice in the protein crystals, which they attributed to interactions between water molecules and the protein surface.

Taylor and Glaeser also developed technical solutions for specimen handling at cryogenic temperatures. They showed that cooling results in improved resistance to radiation damage, such that longer exposure times or larger electron intensities are possible (29,30). In other words, they concluded that cooling the specimen would increase the information content.

### Cryo-EM structures at high resolution

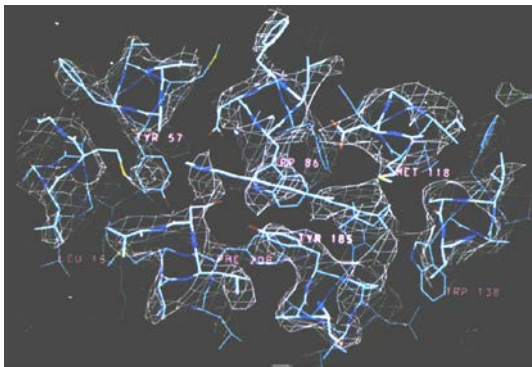


Fig. 2. An atomic model of bacteriorhodopsin superimposed on a slice through the 3D density map. Image from (31).

The year 1990 marked a critical milestone when Henderson and colleagues (31) showed for the first time that it is possible to obtain high-resolution structures of biomolecules using cryo-EM through averaging over many copies of the same object (**Fig. 2**). In their experiment, the signals originated from many bacteriorhodopsin molecules in a 2D crystal. The study showed that at cryogenic temperatures, the consequences of radiation damage could be limited to retain the information content sufficiently to reveal positions of

amino-acid side chains of the protein. In subsequent years, a similar approach was used to determine high-resolution structures of, for example, the light-harvesting complex (32,33), the tubulin dimer (34) and aquaporin (35).

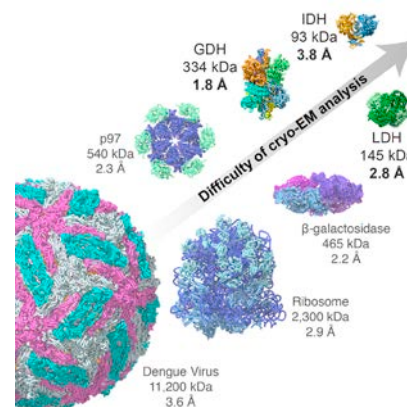
In their pioneering study, Henderson and colleagues used several electron microscopes around the world to optimize the data quality (31). They identified a number of technical limitations of these microscopes, as well as challenges associated with sample preparation, which at that time conspired to limit resolution. Henderson concluded that no microscope was perfect at that time and argued that specific technical and specimen-preparation improvements would facilitate development of cryo-EM to a general technique: *"This would then turn the technique we have been using into a routine and quick method, able to be used on many more difficult specimens, eventually including non-crystalline molecular assemblies"* (31).

The first high-resolution model of bacteriorhodopsin (31) was based on analysis of millions of protein molecules in a 2D crystal, which allowed the spread of the total electron dose over a large number of particles.

The analysis of a large number of molecules in the 2D crystal is equivalent to averaging directly in the microscope. For non-periodic assemblies of symmetrical particles, the signal-to-noise ratio can be increased by averaging over the asymmetrical units. However, for the general case of non-periodic asymmetrical particles, the challenge was to determine the position and orientation of each particle in an image from weak signals. Once this could be achieved, averaging would be possible. However, such analyses would require computer power well beyond that available in 1990 (36).

Large virus	300 M	Yes
Small virus	11 M	Yes
Ribosome	3.3 M	Yes
	1.4 M	Yes
Multimeric enzyme	420 K	Possibly
	180 K	Possibly
	52 K	Possibly
Small protein	18 K	No
Very small protein	7 K	No

**Fig. 3.** Extract from Table 2 in (37), which addresses the question: can single molecule alignment be carried out in practise? The answer is given to the right for a number of example proteins, listed to the left, with molecular weights (Da) in the middle column.



**Fig. 4.** Examples of structures determined using cryo-EM as of May 2016 [image from (38)]. The figure illustrates the conclusions from (37). Note that the smallest protein (64 kDa) determined to date using cryo-EM is haemoglobin (39).

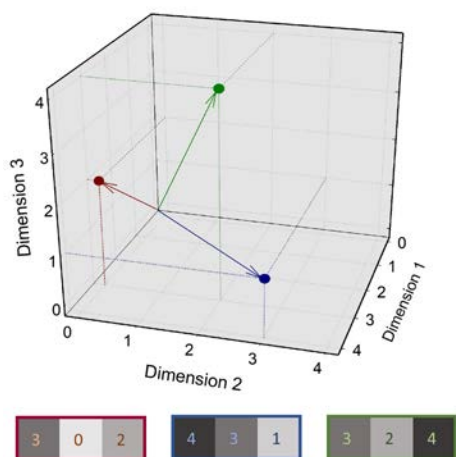
Five years after the publication of the high-resolution structure of bacteriorhodopsin, Henderson presented a quantitative analysis of the challenges needed to be overcome in order to determine atomic-resolution structures of non-crystalline molecular assemblies (37). He concluded that by using low-intensity, non-destructive electron irradiation in phase-contrast electron microscopy, it would be possible to determine the 2D position and 3D orientation of individual particles, given a sufficiently high molecular weight. The information would allow averaging ensembles of randomly distributed particles, thereby eventually reaching atomic resolution. The conclusion from Henderson's work was that, assuming a molecular weight higher than ~50 kDa (**Fig. 3**), it should be possible to align and average a reasonable number (~10<sup>4</sup>) of particles to determine structures at atomic resolution (~3 Å; see **Fig. 4**). During the coming years the predicted number of particles necessary to reach this resolution was revised to smaller numbers (36,40), and Glaeser presented an analysis that suggested that the size limit could be adjusted to ~20 kDa (36). An analysis along the same lines, but less detailed, was also presented in an earlier paper (41).



## Ensembles of single asymmetric particles in solution

A fundamental problem in studies of unstained, non-crystalline, asymmetrical, randomly oriented particles in solution is "*the alignment of features that are only faintly visible on a noisy background*" (20). In the mid-1970s, Frank addressed this problem in a study that became in many ways the starting point for future developments (20).

Frank and colleagues presented a method for aligning low-dose images of individual molecules using cross-correlation functions (20,41,42). A quantitative analysis of the problem was presented in 1977 (41). The analysis concluded that it would be possible to locate randomly positioned particles using non-destructive electron doses. Consequently, the implication was that it would be possible to average images of many radiation-sensitive particles to eventually obtain high-resolution data. The feasibility of the approach was illustrated in studies of negatively stained glutamine synthetase (43).

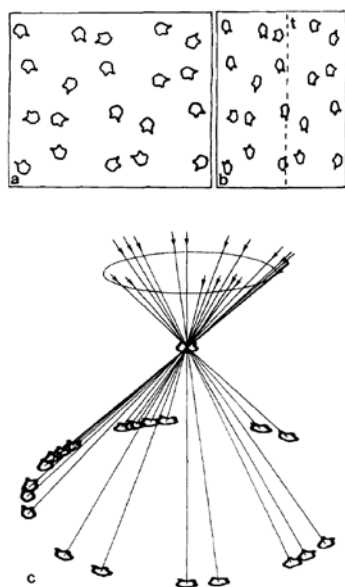


**Fig. 5.** Graphical representation of a method for particle sorting (44,45). Each particle image (here in different colours) is represented by the intensity of  $n$  pixels, here illustrated by only 3 pixels. The intensity of each pixel is translated into an integer number and the particle is illustrated by a vector (modified from a figure prepared by J.L. Rubinstein).

Each particle image, represented by the intensity of  $n$  pixels, is described by an  $n$ -dimensional vector (**Fig. 5**). Multivariate statistical analysis is used to sort the vectors, which appear in clusters. Each of these clusters is assumed to represent a 2D projection of a particle with specific orientation and structural properties. The approach could be computerized to automatically sort images based on subtle differences. Particles within each cluster are assumed to be sufficiently similar to allow averaging to increase the signal-to-noise ratio.

For a non-crystalline specimens consisting of uniform particles, the challenge is to determine the position and orientation of each particle, i.e., the five parameters that determine their 2D position in the plane and their 3D orientation. However, biological samples are rarely structurally uniform and may contain impurities. Therefore, another requirement is to identify potential structural sub-states, to identify different types of particles for heterogeneous samples and, in the case of stained particles, to identify differences in the negatively stained structure. In 1981, Frank and Marin van Heel presented a method that allows sorting of particle images into classes based on their orientation, as well as their structural features (44,45).

Each particle image, represented by the intensity of  $n$  pixels, is described by an  $n$ -dimensional



**Fig. 6.** Random conical reconstruction. (a) Randomly oriented particles; (b) projection of (a), tilted by 50°. The images in (b) form the conical tilt series, illustrated in (c) as a single particle that is randomly projected with all directions lying on the surface of a cone. Image from (47).

Another challenge is to determine how the 2D classes are related to each other in 3D for a given structural sub-state. A general method to determine the relative 3D orientation from classes of 2D projections of asymmetrical particles was presented by Frank and Michael Radermacher in 1986-1987 (46,47). The method is called Random Conical Tilt; it is based on the general idea of obtaining 3D information from 2D projections presented earlier by Frank and colleagues (43), combined with the application of a tomographic conical tilt series, described by Radermacher (48) (**Fig. 6**).

Frank developed many of the important mathematical tools used for image analysis, which form the basis for single particle cryo-EM. He gathered them together in a suite of computer programs called SPIDER, making them readily available and useable for the scientific community (49,50).

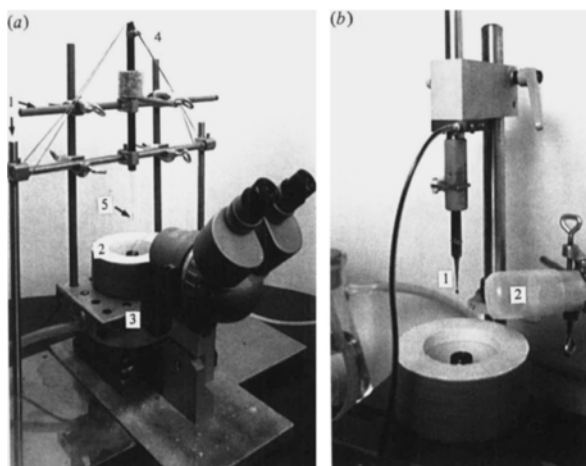
## A sample-preparation method for cryo-EM

As discussed above, cooling was expected to solve many of the complications that limited the use of electron microscopy for structural studies of biomolecules. Problems associated with

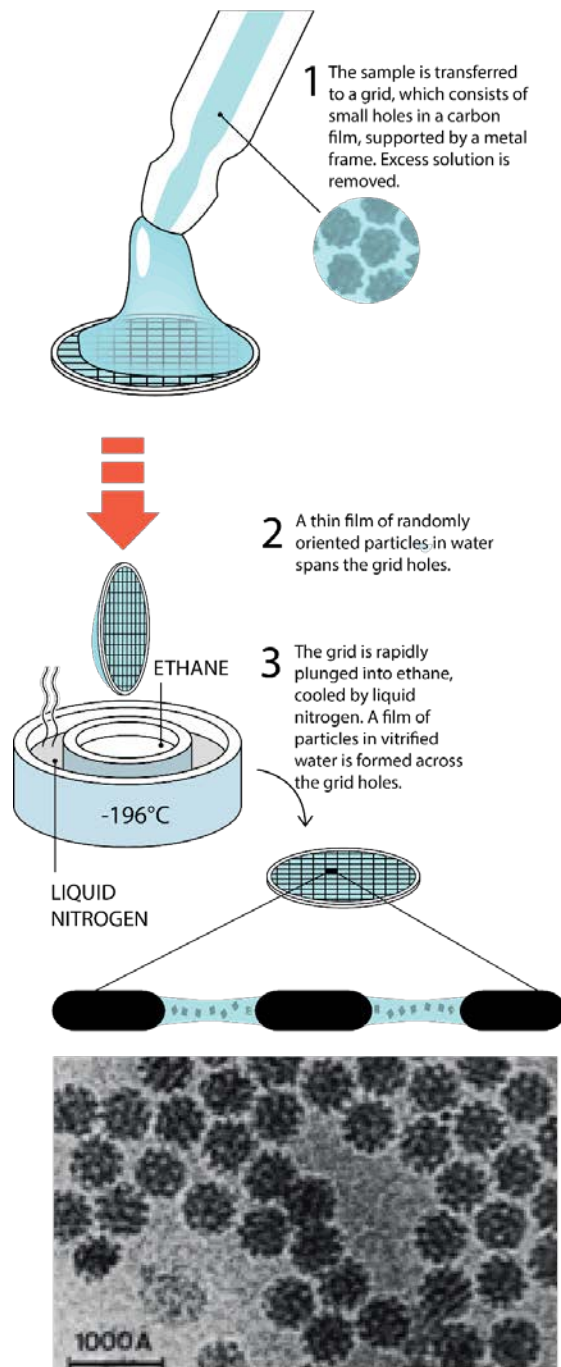
formation of crystalline ice could, in principle, be overcome by cooling liquid water into a vitrified state.

However, before 1980 whether bulk water could be transformed into a vitrified solid state was still controversial because theory predicted that the required cooling rate would be practically unattainable. The phenomenon had been demonstrated, but only for condensation of water vapour at cold metal surfaces (51,52).

In 1980 the discussions were brought to an end with the demonstration that vitrified water could be formed by



**Fig. 7 Plunger for freezing.** A simple (a) and more elegant (b) freezing apparatus equipped for preparing thin vitrified layers of suspensions. Image from (55).



**Fig.8.** Sample-preparation procedure for cryo-EM. Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences. The image of a Semliki Forest virus suspension at the bottom is from (57).

rapid cooling of micrometre-sized droplets of bulk water (53). It is also interesting to note that vitrified water may be the most common form of water in the universe (54). In 1981 Dubochet and Alasdair McDowell finally presented a method that allowed formation of a film of non-crystalline solid water on a specimen grid for observation in the electron microscope.

Water was sprayed on a carbon film mounted on a grid, after which the grid was rapidly immersed in liquid ethane or propane at about  $-190^{\circ}\text{C}$ , maintained by cooling in liquid nitrogen (56). The thin layer of vitrified water was shown to yield nearly uniform absorption of electrons in the cryo-EM. The amorphous structure of water was converted into a crystalline form upon warming to about  $-140^{\circ}\text{C}$ . Dubochet and colleagues noted that vitreous ice could be maintained around the specimen for extended times if the temperature was kept below  $-160^{\circ}\text{C}$  (55,57). In the years following, Dubochet and colleagues developed the sample-preparation method further (**Fig. 7**) and presented detailed studies of pure water, aqueous solutions and suspensions of bacteriophages, purple membranes and DNA at cryogenic temperatures (58-60).

The full potential of Dubochet's sample-preparation method was realized in 1984, when the group presented electron micrographs of virus suspensions, cooled using an improved method that allowed preparation of thin, unsupported water layers in the vitrified state (55,57). The new technique made it possible to prepare unsupported water layers that could

be made sufficiently thin to allow rapid vitrification, but thick enough to accommodate a single layer of randomly oriented molecules or molecular complexes in their native state (**Fig. 8**).

Dubochet and colleagues showed that the sample-preparation method is generally applicable in cryo-EM studies of other biological particles as well (55,61). The resulting images showed an impressive contrast. Finally, in the beginning the 1980s, Dubochet had solved the problem that had cast a shadow over the first 50 years since the invention of the electron microscope: “*the most abundant constituent of living things, water, has invariably been excluded*” (61). The new preparation method was immediately adopted by Dubochet’s colleagues and it is now used universally within the cryo-EM field, both in studies of assemblies of single particles and in cryo-tomographic studies of single objects.

### **Recent technical developments**

The latest technical developments that made the recent breakthrough possible was the introduction of new electron detectors in electron microscopes. These detectors are constructed from Monolithic Active Pixel Sensors, based on Complementary Metal Oxide Semiconductors, known as CMOS technology. Early reports on the use of these sensors for detection of electrons were presented around the mid-2000s (62-66), but these detectors had already been used as a tool in studies of charged particles in other research fields, such as astronomy. The sensors, referred to as Direct Electron Detectors, were made widely available in cameras for electron microscopes in 2012-2013.

Silica-based charge-coupled devices (CCD) had been used earlier; however, for technical reasons these detectors were not used to monitor electrons directly. Instead, the electron flux was first converted into light, which introduced noise to the signal. As a result, in many cases, film was a preferred choice when analysing high-resolution data.

The new Direct Electron Detectors presented an improvement in their ability to detect high-energy electrons at low intensities with less noise than film. Another advantage is the speed at which these cameras operate. In many cases it is possible to record a “movie”, which is used to compensate for specimen motion caused by electron irradiation and temperature drifts. Electron counting has also become possible (67,68).

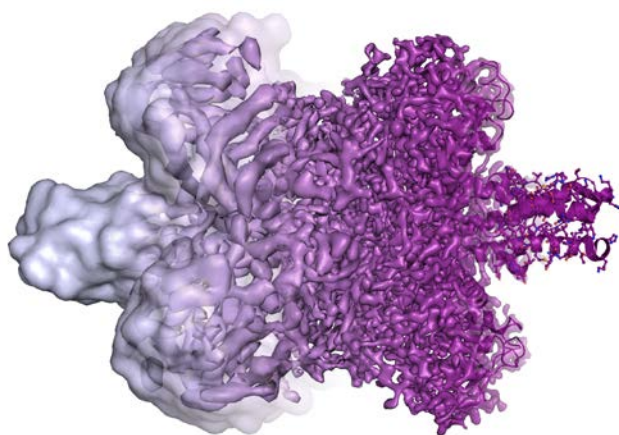
Collectively, these improvements in detector technology resulted in a dramatic increase in the signal-to-noise ratio and spatial resolution (67,69-72). Other technical innovations, such as the field emission gun (electron source) developed by Albert Crewe and stable cold stages [e.g. (73)] were also key to the more recent developments.

Another technical innovation introduced recently is the Volta phase plate (74), which potentially can solve problems of phase correction, encountered when using defocusing to obtain phase contrast in the electron microscope (24). Automation of data collection (75) has also significantly advanced the field.

Recent and ongoing improvements in image-processing methods and computer programs have also been essential for the current developments. For example, maximum likelihood algorithms (76,77) became particularly important in electron microscopy when better resolution was achieved using the new electron detectors.

## Summary

The first high-resolution structure, determined using cryo-EM, was presented in 1990. A decade passed before high-resolution structures of helical and icosahedral particles were imaged, determined based on analysis of data recorded on film. After the introduction of the new Direct Electron Detectors in 2012-13 (see **Fig. 9**) and the first reports of *de novo* atomic structural models of smaller single particles, such as that of the membrane protein TRPV1 ion channel (78), cryo-EM has very rapidly become a major new tool in structural biology.



**Fig. 9.** The resolution progression of cryo-EM, illustrated by a representation of glutamate dehydrogenase with an increasing level of detail from left to right. For a protein of this size, 334 kDa, the 1.8 Å resolution to the right (38) could only be achieved after 2012/13. After an image by V. Falconieri (see ref. 38). Illustration: © Martin Högbom, Stockholm University.

It is captivating to think about the amount of time that has passed before we could get to this point. About six decades after John Kendrew's and Max Perutz's pioneering crystallographic work on myoglobin and haemoglobin (Nobel Prize for Chemistry in 1962 "for their studies of the structures of globular proteins"), and four decades after the first developments that laid the groundwork for single-particle cryo-EM, a high-resolution structure of haemoglobin in solution, determined using cryo-EM, was presented (39).

Single-particle cryo-EM is unique in that it does not require crystallization, uses very small amounts of material, and covers a wide range of sizes, from particles the size of haemoglobin (64 kDa), to very large particles up to several megadaltons. Cryo-electron tomography is used to determine structures of even larger objects, including organelles and cells, with the potential of

being able to obtain high-resolution information from molecules or complexes *in situ* (79,80). Thus, with the recent developments, cryo-EM extends the possible size range for structure determination in solution, from cells and organelles to molecular complexes, molecules and the atoms that build these molecules.

But cryo-EM is not only about static structures. Because sample preparation for cryo-EM involves instant cooling of a solution, the contents of the solution can be systematically varied; integral membrane proteins may be studied in a near-native environment; and the particles may be trapped in structural sub-states or even in action, for example, while an enzyme catalyses a chemical reaction. The data may offer functional information: structural changes may be monitored and free-energy landscapes determined (81-86).

These recent developments will certainly be followed in time by others, both in regard to technology and applications. Perhaps in the future we will be able to obtain high-resolution structural information of molecules, as well as to observe interactions and dynamic processes as they happen, inside cells or organelles.

During his journeys in 1968, the fictional Mr. Tompkins asked his guide about the electron microscope: "Is it true that it is so powerful that you can see atoms with it?" The guide smiled and declared: "Not quite. But you can see the larger protein molecules, and this is impressive enough."

The view that the technique would not achieve much better resolution was shared by many researchers in the field, even in more recent times. Perhaps these accounts illustrate the substantial efforts needed to bring cryo-EM to today's level and why these developments have been referred to by Werner Kühlbrandt as "the resolution revolution" (87).

This progress would not have been achieved without the contributions of the Laureates. Jacques Dubochet developed methods for preparation of samples for cryo-EM studies of biomolecules in water. Joachim Frank developed methods for structural determination of biomolecules from analyses of ensembles of particles in solution. Richard Henderson demonstrated that it is possible to obtain atomic resolution structures of biomolecules using cryo-EM.

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Professor of Biochemistry, Stockholm University  
Member of the Nobel Committee for Chemistry



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