Early cryo-electron microscopy

Nobel Lecture, December 8, 2017 by Jacques Dubochet
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IN THIS VERY SPECIAL moment my first feeling is thankfulness. It goes out to my late parents, to my family that I have the pleasure of seeing in the audience, to my scientific colleagues, to whom I owe being here, to my friends, and to all those who contributed to making me who I am, here and now. I want to pay a special tribute to Prof. Édouard Kellenberger, my "doctor father" and lasting friend, who taught me how to be a scientist and passed onto me the sense of responsibility that should be associated with this profession. My special thanks also go out to Sir John Kendrew, first General Director of the European Molecular Biology Laboratory (EMBL) in Heidelberg, who gave me the chance to conduct, under ideal conditions, our ambitious project on water in electron cryo-microscopy (ecm).

WHY ELECTRON CRYO-MICROSCOPY?

Like any living organisms, we are a bag of water, formed from billions of cells which all are small bags of water. Since air is not transparent to electrons, an electron microscope must operate under vacuum – which means that any observed biological specimen must be dry. This is not good. The original structure can't be preserved in these conditions. When water is removed, floating molecules stick to each other. Skilled microscopists know how to minimize the damage, but they will never prevent some forms of aggregation since "fishes never fly". Even objects supposed

Figure 1. Prof. Édouard Kellenberger, Sir John Kendrew.

to be resistant, like bacteriophage T4, look terrible when they are dried on a solid surface without particular precautions (figure 2a). For decades, electron microscopists have invented methods improving the structural preservation of every possible dry specimen. Negative staining has proved especially effective, as the subtle details visible on the micrograph demonstrate (figure 2b). Freeze-drying is a bit more complicated, but also has its advantages (Figure 2c). Nevertheless, it is obvious that the head of the virus does not look healthy.

Since the end of the 60s, I was among those working hard to find better methods for preparing and observing delicate biological specimens. At

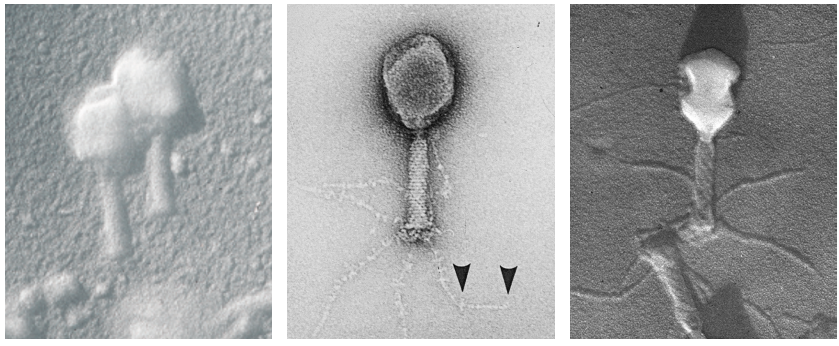

Figure 2. T4 bacteriophages prepared by different methods. a) Direct drying. b) Negative staining; the specimen is in a solution of heavy metal salt that forms a protective coat around the particle when water evaporates. c) Freeze-drying; a thin layer of suspension is frozen on a supporting film. Ice is removed by sublimation under vacuum.

the time, my hero was Nigel Unwin. I was impressed by his creativity and skill. Beside the similarity of our research's direction, I discovered – having been invited in his home the first time I was in Cambridge – under the bed, a self-made telescope of the same type that the one I had built myself in my late adolescence. Would the Nobel Prize by-law allow four persons to share the prize, I do believe that we would be standing here together. In the 70s, Nigel had a brilliant idea. He realized that drying a biological specimen in a heavy metal salt was not the best environment for preserving a delicate structure; friendlier surroundings would be better. He tried to do it in sugar. It worked. Of course, the contrast in sugar is much lower than in metal, but Nigel realized that contrast is not the limitation – that is the signal-to-noise ratio. This can be improved by means of methods used in X-ray crystallography, taking advantage of the redundancy of the information in a crystal. He joined forces with his friend and colleague Richard Henderson, an experienced crystallographer, and together in 1975 they solved the first 3-dimensional structure of a membrane protein (Henderson and Unwin, 1975).

Bob Gleaser is another person of great importance for me. I worked in his steps for a good part of my PhD, and it was a micrograph he published in 1976 (Taylor and Glaeser, 1976) that redirected my working plans. It was a sample of broken bacteria containing a rich collection of their various substructures. Some of them were also subjects of our own research. This micrograph was special because it was a thin frozen layer of the aqueous sample observed at –170°C in a specially cooled specimen holder. The specimen was in ice and the biological material was more beautiful than anything I had ever seen before. I was immediately convinced that cold water was the future. Two years later, Sir John Kendrew offered me a position as group leader at the newly formed EMBL for a project entitled "How to Deal with Water in Electron Cryo-Microscopy". As it has been explained elsewhere (Dubochet, 2011), it didn't start well at all. But the continuation proved to be better.

The problem with water is that it crystallizes into ice when it is cooled at a temperature in which it does not evaporate in the vacuum of the electron microscope. So, we had to learn more about water, cooling, freezing, and observing. We tried everything we could think of, and learned from all our predecessors in the field. As it turns out, we started experimenting with the sophisticated machine presented in Figure 3 (a copy of it is presently exposed in the Nobel Museum). On the right, not visible, is a nebulizer throwing a stream of microdroplets of water through a small slit in the cupboard. The mobile tweezer, above the dewar filled with liquid nitrogen at –188°C, is holding a grid covered with a thin specimen supporting film. We let the tweezer fall and the grid, having harvested some droplets while crossing the stream, is immediately frozen. The frozen ice

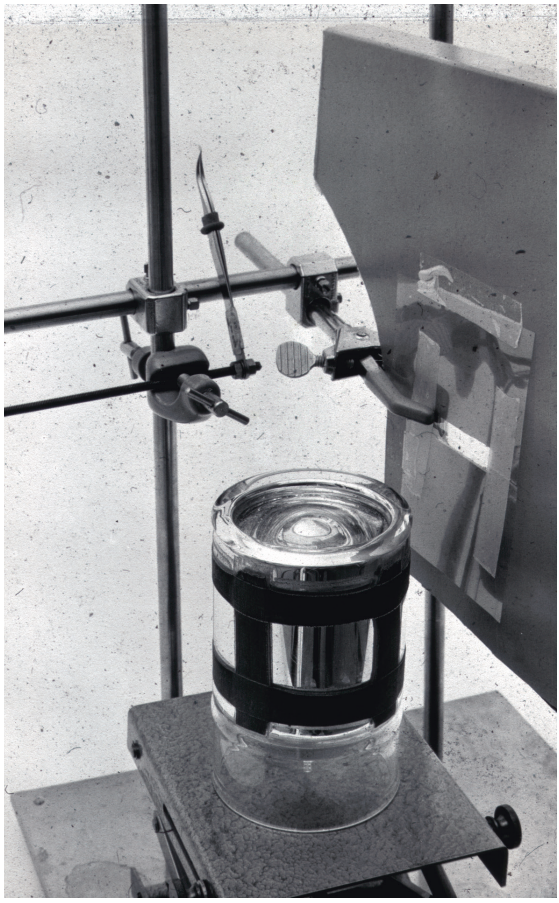

Figure 3. The apparatus for freezing water microdroplets.

droplet has the characteristic aspect shown in Figure 4a. One day, my colleague Alasdair McDowall (Figure 4b, inset) decided to place a little beaker in the liquid nitrogen dewar and condense in it liquid ethane, because it was known that it is a better coolant than liquid nitrogen. He called me to the microscope, something unexpected was there (Figure 4b). It was a "frozen" microdroplet; it was not ice, it was amorphous. We didn't know what it was. We let the specimen warm up slowly – at that moment it was at about −160°C – hoping that the evaporation of the droplet would tell us more about its nature. Suddenly, at −135°C, in a few seconds, the droplet turned into a multi-crystal of a substance we knew well from previous experiments. It was cubic ice, a form of ice which is typically formed by condensation of water vapor at low temperature. The conclusion was obvious. We were seeing ice originating from an amorphous substance: it was vitreous water. I told Alasdair: "Aha! We have something great!"

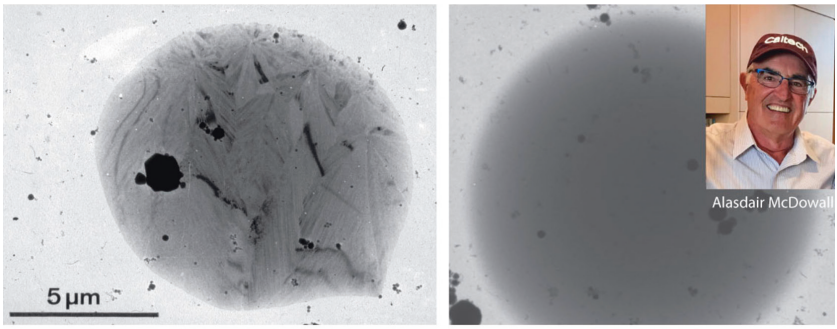

Figure 4. a. Frozen ice microdroplet. b. Vitrified water microdroplet.

The trouble was that vitrification of liquid water should have been impossible. This was demonstrated on solid thermodynamics grounds over decades of previous work. Basile Luyet, acknowledged father of cryobiology and Catholic priest in the congregation of St-François de Sales was among the major contributors to this body of work. I like Basile Luyet because of his strange combination of strict Catholic faith with uncompromised scientific mindset. I also like him because he was born in Savièse, a village in the Wallis Alps of Switzerland to which he remained attached all his life. It is only a few kilometers away from the village of Nendaz where I spent my first school years, at a time when understanding things of nature became important to me.

As a consequence of the accepted impossibility of vitrifying water, the report of our observation was rejected from publication. The editor was doubly wrong.

Firstly, because at the very moment our article was rejected, the same journal had in press the article of an Austrian group demonstrating that vitreous water can be obtained by rapid cooling of the liquid. Their experiment was similar to ours but they used X-ray diffraction to demonstrate the nature of the observed substance and its transformation into ice upon warming (Mayer and Bruggeler, 1980).

Secondly, they were also wrong because – as we reported – vitrification is rapid, reproducible and easily repeated.

So why is it possible to obtain vitreous water when it should be impossible? Is this one more illustration that science sometimes fails? Not so fast! The work of Luyet and of those of the field is solid, and thermodynamics should not be taken lightly. At present, we still don't really understand the nature of the vitreous water we observe. We know that it is not simply immobilized liquid water, but some other form of amorphous solid. The science of water still has shadowed regions. I can imagine that, when the light comes, it will have consequences on a larger scale – for

biology also. For now, we are pleased to observe that biological objects vitrified by rapid cooling seem to be indiscernible from those floating in good bona fide liquid water. Electron cryo-microscopists feel safe in their knowledge for now, but they prudently keep an eye on the real nature of vitreous water.

Knowing how to vitrify a droplet of water is one thing; preparing a biological sample for biological observation is another. The major problem comes from the high surface tension of liquid water, which makes water droplets spherical. Spreading a thin layer of liquid on a supporting film requires that the interaction's energy between the drop and the surface must exactly compensate the surface tension of water. I was an expert on how to treat supporting film for optimal wetting. We were combining this knowledge with our newly acquired competence in vitrification. At that time, late Dr. Marc Adrian (Figure 5 inset), a French microscopist of great culture and strong mind, had joined the group. He didn't like our subtle and poorly reproducible spreading procedures. He wanted to get rid of the supporting film altogether. I tried to discourage him, but Marc was not one to easily give up. A while later, he came up with the kind of image shown on Figure 5. It was a vitrified layer of a Semliki Forest Virus (SFV) suspension stretched over the 18μm holes of a grid. There is no supporting film, just a thin layer of suspension with perfectly preserved virus floating immobilized in their vitrified aqueous medium. Indeed, the ideal specimen for electron microscopy observation. Adrian's method is simple. The grid is held on a tweezer, itself mounted on a plunger. A drop of suspension is put on the grid and most of it is then sucked away with a blotting paper. This takes about one second. The surprising thing is that the last fraction of a micrometer takes another full second before it breaks and vanishes. This leaves ample time for the operator to liberate the plunger and let the grid fall freely into an ethane beaker some 10 cm

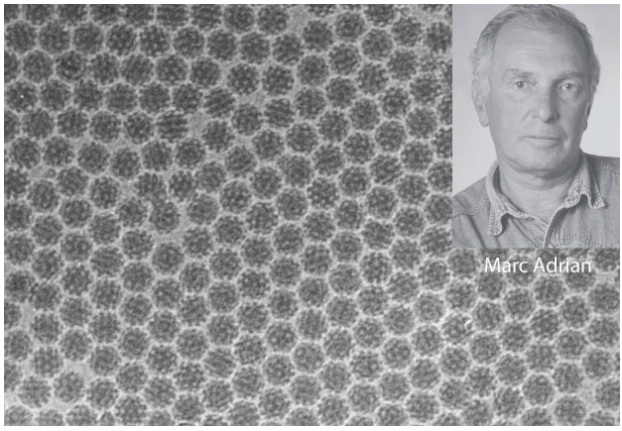

Figure 5. Unsupported thin film of vitrified suspension of SFV. Inset: Marc Adrian

below. The preparation is simple, it only takes a few seconds and it is easily reproducible. It came as no surprise that the rumor of this elegant preparation method spread rapidly. It was a great time in our laboratory. Water specialists, some of them quite incredulous, came to observe the strange phenomena of vitrification. We learned a lot from them. Electron microscopist colleagues wanted to adopt the method. The first electron cryo-microscopy course was organized. The result was broadly published (Adrian et al., 1984). One day, I got a phone call from Pierre-Gilles de Gennes, the world leader on entangled polymers and spreading viscous fluids. One of his books was a difficult read I kept on coming back to, time and time again. That early morning, he was teasing me. "I am sure that you do not know why your thin layer can survive the final step of the preparation!" He was right. He explained that, in order to break, the two surfaces of the thin layer must fuse together and entropy prevents that – for a moment. He could even articulate a number: one second.

More than thirty years later, the basic principle of Adrian's method is still being used, unchanged. The bare grid was soon abandoned for a grid covered with a film with μm-sized holes. The biological suspension is then stretched through the holes. Nowadays, only the older generation is still using a manual plunger, as full automatic devices are making cryo-specimen preparation simpler and more reproducible. But democratization has a price.

Our results were soon published. When he saw the micrographs of his pet adenovirus in unprecedented details, Lennart Philipson, who succeeded Sir John Kendrew as General Director of the European Molecular Biology Laboratory (EMBL), became convinced that this project by physicists about water was valuable and so it gained his full support. We joined forces with R.H. Vogel and S.W. Provencher, specialists in 3-d reconstruction from 2-d images, and in 1986 we published a 3-dimensional model of the SFV at 35 Å resolution (Vogel et al., 1986) (Figure 6).

Building on previous work and continuously improved by the creative efforts of many scientists – the long-lasting efforts of Joachim Frank for 3-dimensional reconstruction of single particles were of seminal importance – electron cryo-microscopy progressed smoothly throughout the years. The thirty-five Ångstrom of 1986 was well and good but some specialists in X-ray diffraction – for long the dominant method for molecular structure determination – jokingly invented the word "blobology" to describe our work. Thirty years later, 3.5 Å is achieved on a nearly routine basis. Who could imagine this in the 80s? Richard Henderson was perhaps the only one who had this clear vision. He worked continuously to make it become real.

Three and a half Å is certainly not an impressive number for non-specialists. Nevertheless, everyone can understand that the resolution was

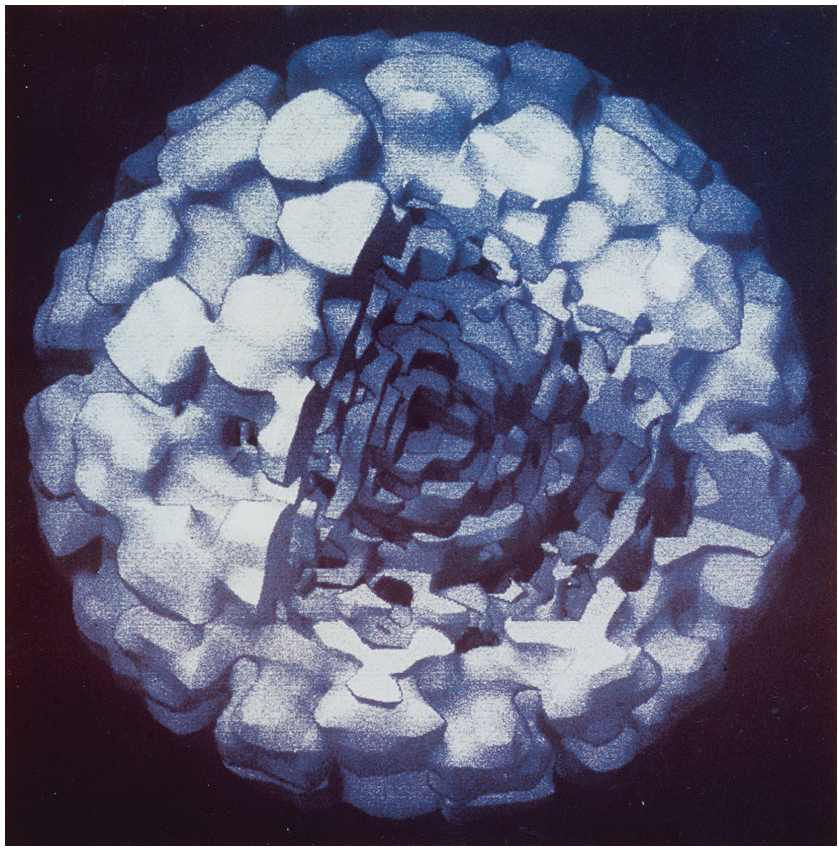

Figure 6. 3-dimensional model of a SFV at 35 Å resolution. Nature 320 (6062), 10 April 1986.

improved by a factor of 10 since the 80s. This means that the volume element resolved at present is one thousand times smaller than before; the density of information that can be harvested from the specimen is now multiplied by one thousand. This is truly a remarkable achievement. Bravo!

But the real breakthrough came from the fact that, around 3.5 Å, atoms become visible. Or, in other words: blobology becomes chemistry. This is the reason why three biophysicists who never thought of themselves as good chemists are gratified with a Nobel Prize in Chemistry. This is not because we have reached our level of incompetence, as promised by Peter's principle; it is a testimony to the unity of science. Physics, biology, chemistry; all is just science.

At present, electron cryo-microscopy has not yet brought an important result that could be translated into practical applications whether in med-

icine or in technology, but this will come, soon! Chemistry is a powerful science. When the arrangement of atoms can be visualized, the possibility to act on them is not very far from our reach. For example, will it soon be possible to prevent the pathological entangled binding of proteins that seems to be associated with Alzheimer's disease and numerous other neurological disorders? Many of us would be interested in such progress "for the greatest benefit to mankind", along the line of Alfred Nobel's expectations.

And science will continue. It will take time to explore the brain. We may understand how we think. Perhaps conscience will emerge. This is knowledge without limits.

But knowledge also has its practical consequences. It shapes our lives.

I got my first personal computer in 1984 as Adrian's method was being implemented. Nowadays, billions of people are sitting in front of a computer screen for the major part of their days, and communication between individuals is fundamentally changed.

My grandfather's father was living in scarcity. He was never sure he could bring home the minimum required for a decent life for his family.

=> Now we are submerged with excess
=> and the world's climate is collapsing
=> as is the glacier just above our mountain hut.

Figure 7. Extraordinary ice collapse in the glacier of Ferpècle (Wallis, Switzerland). Photo: Gerard Stampfli.

Five hundred years ago, François Rabelais wrote,

Science sans conscience n'est que ruine de l'âme.
Science without conscience is but the ruin of the soul.

The problem is not new but now, it is urgent.

What can we do?

One thing is for certain: we scientists must come down from our ivory tower and be involved in the society for which we produce knowledge. That knowledge can have equally good or bad consequences, and we must become more aware and responsible.

This is the reason why, more than 20 years ago, we introduced a compulsory curriculum in our university: "Biology and Society". We want our students to be as good citizens as they are biologists.

This is good, but it is not enough.

How can we be as good in using our knowledge for the well-being of all as we are in producing it?

I don't know the solution, but I know the value of knowledge. It is our most precious common good. We must protect it, develop it and make the best of it for the well-being of mankind, now and for future generations.

Imagine,

It's easy if you try.

Imagine, for example, that we think about health.

Imagine that we empower the World Health Organization, United Nations WHO, with all we know about medicine and medical treatments, and trust this institution with the duty and the competence to use this knowledge for the well-being of all. Of course, we will give those who produce the knowledge the rightful reward for their efforts.

It isn't hard to do.
Imagine...
You may say I'm a dreamer
But I'm not the only one
I hope someday you'll join us
And the world will live as one
John Lennon

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