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History of Cryo-EM

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Richard Henderson discusses Jacques Dubochet's, Joachim Frank's and his contributions to the development of electron cryo-microscopy. See video at https://youtu.be/ZnxCnUmtKGU

Mejd Alsari (MA). One of the big questions since the early days was how can we resolve the atomic structure of biomolecules using TEM? Can you briefly summarize the aspects that needed an improvement in order to go from a resolution of 7 Å to 3.5 Å?

Richard Henderson (RH). In the 1930s we had the first electron microscope.[1,2] Everything about it was very crude. The electron source was crude, the lenses were crude, the way you recorded the images was crude. You had to have this heavy metal stains and it was very low resolution, a sort of very distant view of the molecules.[2,3] You couldn't see atoms. You could barely see biological molecules at all. You could see viruses and bacteria because they were very big. Many things from 1930 to 1950 had to be improved. If we jump to the current day, now we have electron cryo-microscopes that work at 1-2 Å resolution. The underlying theoretical limits are the same. All of the improvements were practical ones.

For example, if you want to put a specimen into the vacuum of an electron microscope and cool it down to liquid nitrogen temperature (-196 °C), so that the water molecules don't move around and the amorphous ice doesn't recrystallize into normal hexagonal ice, you have to have a very cold temperature. If you did that right up to about 1980 the specimen would immediately get covered with a contaminating layer of ice that was extracted out of the bad vacuums of the microscope. So the very first improvement was to get good vacuums and that didn't come in until about 1980. Jacques Dubochet was one of the first people who realized that and had a microscope in 1980.[4,5]

At that point you have to take electron micrographs, and once you cool the specimen to liquid nitrogen temperature often the liquid nitrogen is boiling so you get vibrations and the images were blurred. So the cold stages that worked, now, and had a good vacuum, didn't give high resolution so then you needed cold stages.

Once we had a vacuum and then we had cold stages it turned out the electron source, which was usually a bent piece of tungsten wire, just like in an old-fashioned tungsten electric light bulb, did not give a very bright source of electrons. In the electron optics of the focusing, that meant that the so-called coherence of the illumination was not high enough. Then they had to go from tungsten sources to field emission guns, which are a thousand times brighter, with a much sharper point, which give much higher resolution data. That came in the 1990s.

After we had vacuum stages and field emission coherence sources it turned out the detector needed to be improved. Until about 2010, old-fashioned photographic film were the best way of recording images. In the last 20 years or so the big improvement has been in the detectors and that came in about probably six or seven years ago.[6-10]

Then all of the different barriers had then been overcome and you could then start taking images which were much more beautiful. Then all the people who did computer-based image processing developed better programs to deal with better images with all the better microscopes and so on.[11] That's made a big revolution.[12]

Figure 1 | Richard Henderson, group head at the MRC Laboratory of Molecular Biology, Cambridge, and Mejd Alsari, post doctoral researcher at the Cavendish Laboratory (University of Cambridge).

Now the electron cryo-microscopy has become the dominant method in structural biology. If you open the journals now there's still a very large amount of work done by NMR and X-ray crystallography, but all the difficult projects and all the ones that have a high profile are all being done by electron cryo-microscopy now, because all of these technical problems have now been largely overcome. There are still quite a few more problems to be solved that will make the method even more powerful. It's still on a rising level of expectation.

MA. What about the issues with the negative staining method and other sample preparation techniques? Why researchers were interested in vitrified water and what were Jacques Dubochet's breakthroughs between the 70s and 80s?

RH. Jacques Dubochet started out as a young research student in Switzerland probably about 1970 and I think he always knew that there was great potential in developing methods further. He was recruited to the European Molecular Biology Laboratory (EMBL) in Heidelberg in 1978 by John Kendrew, who used to be our head of division here in structural studies, here at the Molecular Biology Laboratory (LMB) in Cambridge.[5] In 1978, John Kendrew's (the director of the EMBL) and Jacques Dubochet's (as a young group leader) goal, which they stated before any work started, was to develop methods of making frozen biological specimens and to develop cryo-microscopes that would work with frozen specimens. So they put a lot of resources, effort and money from the EMBL into that.

From 1978 probably till 10 years later, when Jacques Dubochet went back to Switzerland, he was there for nine or ten years, they developed two things. They developed one of the very first electron cryo-microscopes that were essentially homemade with some commercial components. For example they had a very large liquid helium cooled superconducting objective lens from the Siemens

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company in Germany that used to make electron microscopes. They put that into a Zeiss electron microscope. Zeiss was another German company. These companies do not make electron microscopes anymore, but in 1980 they built a microscope like that.

In parallel, Jacques Dubochet and his group, mainly Alasdair McDowall and Marc Adrian, were studying the properties of water.[13,14] They showed, probably about 1980, that when you cool water to liquid nitrogen temperatures, depending on how you carry out the procedure, you end up with either hexagonal ice or cubic ice or, if you cool it rapidly enough, you could get amorphous ice. The method that they developed was to make a thin film of water, perhaps fraction of a micron thick. They did that by putting a drop of liquid onto one of the electron microscope grids that are 2-3 mm in diameter and they simply blotted it with a piece of filter paper so that the liquid came off but it left a meniscus that was very thin. That was Mark Adrian's filter paper method.[15,16]

Then Alasdair McDowall tried plunging the thin film of water into various media. When you plunge a thin film of water or a metal grid into liquid nitrogen at its boiling point, the liquid nitrogen boils and you get a thin film of gas. So it cools rather slowly and you get hexagonal ice. But if you put the thin film into liquid ethane at a temperature just above liquid nitrogen, let's say -185 °C, it has about 100-150 °C between the freezing point and the boiling point and that remains liquid and it cools it very rapidly. So in a fraction of a millisecond you've gone from water at room temperature down to amorphous ice at liquid nitrogen temperature. That meant that you don't get any crystals, you get the molecules beautifully preserved in this homogeneous medium. In the 1980s Dubochet's group was the only one in the world doing this and he published many papers. But in one review they had 20-30 different biological structures all looking beautiful but not at very high resolution.[16]

So they developed the method and the plunge freeze method is still used today. But instead of doing it by hand, you now buy a machine. There are about six companies that make what's called plunge freeze instruments, in which there's a computer that controls the temperature, the humidity, the plunge freeze rate, the blotting rate and so on. So that was how the method was developed and that is the method we still use. Many people now think there must be a better way of doing it and there are a few people trying this, but at the moment nobody has made a better method.

MA. Let's move to data analysis. How can we reconstruct a 3D structure from single 2D images? Can you talk about Joachim Frank's work on the development of image processing techniques to achieve this?

RH. Joachim Frank was the third person who shared this 2017 Chemistry Nobel Prize.[17] His background was rather different. He came from the core of the German electron microscopy community, having done his PhD in Martinsried (Technical University of Munich for graduate studies at the Max Planck Institut für Eiweiss- und Lederforschung, now Max Planck Institute of Biochemistry)[18] with Walter Hoppe. His background was all electron optics and coherence and things like that. Probably around about 1976 or 1977 he started to think more seriously about computational analysis of images.[19,20] Whereas my transition from being an X-ray crystallographer to being an electron microscopist came through crystals. I went from 3D crystals to 2D crystals. Frank went from electron optics into image processing of single molecules.

Originally, in the 70s, all the single molecules that you could look at were all negatively stained or shadowed. All the early work of Joachim Frank, probably up to about 1990, was done on negatively stained specimens. That allowed them to develop many of the computational procedures whereby you take a 2D image like a photograph and by analysing it in the computer end up with a 3D model of that structure, because each 2D image is a view of the molecule from different directions. They call this, for example, whole body imaging. For example, if you have some medical problem like a cancer, tumour, and let's say that's in your lung or it's a brain tumour or so on, you go into the hospital and they use X-rays to take 2D projection images from all the different angles. When you put those together in the computer, with all those different angles, you get a 3D image. There are various algorithms. Joachim Frank was the developer of some of these algorithms, other people developed other algorithms.[21,22]

The emphasis of Joachim Frank was always on the single particles. Then Dubochet developed the plunge freezing method, also for single particles, but in ice. When these methods were brought together you ended up with single particle electron cryo-microscopy, which is the method that became quite powerful.

Dubochet and Frank contributed, you could argue, different components of what had to be put together to make a more powerful method. Whereas my trajectory came from 3D crystals and X-ray crystallography to 2D crystals and the same kind of electron cryo-microscopy. But it wasn't with plunge freezing, it was just with 2D crystals and so on. My transition again came probably in the 1990s, when it became clear that the real power of electron microscopy is not to use it as a method of getting diffraction patterns, which is what all the X-ray crystallographers and all the electron crystallographers had been doing, but to get images. Images were the most powerful thing.

In the end everyone converged on identifying where the key route to making progress was. Then throughout the late 1990s early 2000s people were focused on writing computer programs.[23,24] Then when the new detectors came in everybody was kind of ready to go. Then from 2013 to 2019 now there's been a sort of exponential growth in the number of people, either new people being trained or structural biologists with other types of skill transferring into the electron cryo-microscopy community.

MA. In 1975 you published a paper in nature titled 'Three-dimensional model of purple membrane obtained by electron microscopy'[25] and fifteen years later you published another paper on the same structure, where you achieved a higher resolution.[26] Can you talk about your work on the purple membrane and how were you able to achieve that resolution?

RH. When I was a younger scientist as a post-doctoral visitor in the USA at Yale, I had worked previously on the structure of enzymes using X-ray crystallography.[27] Many people were beginning to be interested in proteins that were in membranes, but it was not so clear how you could do that work. I did spend a couple of years trying to work on a membrane protein family called voltage-gated ion channels.[28] It was clear then that was going to take very long time. The methods weren't available. Cloning wasn't available. You couldn't purify things. So I was looking for a simpler protein that would be more suitable for structural biology.

Then a group led by Walter Stoeckenius, who was then in San Francisco, had discovered this purple membrane, which has one small protein in it and it's coloured purple because it has, in addition to the protein polypeptide chain, what's called a chromophore, vitamin A aldehyde.[29,30] This is the same thing that's as a sort of dimer in carotene, which makes carrots orange, for example. Half of carotene makes vitamin A aldehyde. One of those molecules is in bacteriorhodopsin and when it binds to the protein it changes from yellow to purple. That protein in the membrane of the bacteria absorbs light, pumps hydrogen ions, protons, out of the cell, and creates membrane potential. That gives the cell an energy source so it can swim around and live.

That was a very good membrane protein that was more tractable, I thought, in 1972-1973. When I came back from the USA to the UK, to Cambridge, to the MRC Laboratory of Molecular Biology in 1973 the idea initially was to make crystals and do X-ray crystallography. But I met another group leader, who is still here, Nigel Unwin and his background was in electron microscopy. He had worked in the department of material science and metallurgy in Cambridge

and then he had come here to try to develop electron microscopy for biology.

We met up and we collaborated for about two years. Without any cryo, it was all at room temperature, we managed to get reasonably good images of the purple membrane with this single protein in it.[25,31] The protein is called bacteriorhodopsin because it resembles, it's not identical, but it resembles the pigment at the back of our eye, in the retina. Rhodopsin is the visual pigment used for human vision. It wasn't bacterial vision, it was bacterial energy transduction.

We got a 7 Å structure, which showed α-helices in the protein, just like they had found in myoglobin in 1957. When we published that 1975 paper we copied the title. The original paper was 'A three-dimensional model of the myoglobin molecule obtained by X-ray analysis'[32] so we said okay 'Three-dimensional model of purple membrane obtained by electron microscopy'[25]. In 1975 we were pleased it was the first membrane protein structure that showed trans-membrane helices. We were very pleased with that.

But nothing that we did, said that the resolution should be only 7 Å. In principle, we put the specimen in the microscope, we take a picture, it should diffract to any resolution you want. We actually spent quite a long time trying to work out why it didn't go to a higher resolution. Because then (with higher resolution) we get the atoms, and we could get the chemistry, the structures, the mechanisms, etc. In the 1970s we actually thought the main thing was maybe that the film wasn't very good. Then after the film we said, "Well maybe the instruments that digitized the image on the film, the film scanners, they weren't so good". We spent years trying different films, building better and better film scanners and so on.

Then we tried two other methods. We tried to instead of taking images we thought we could take diffraction patterns and use the methods the X-ray crystallographers had developed, all indirect, and none of these turned out to be powerful enough.

In the end it was the development of the cryo-microscopes. Dubochet was one of them, several people developed cryo-microscopes. Then through the 1980s we had one here. It wasn't as good. I went to Berlin, Berkeley in San Francisco, and EMBL in Heidelberg, where Dubochet had this liquid helium lens. There were three microscopes and we got images from all of them. They all went into the analysis. Using the very early prototypes, homemade electron microscope, collaborating with these three groups, we finally got enough data to get an atomic model in 1990 (Figure 2). That was 15 years.

During these 15 years we were doing different things at each stage, trying to get to the bottom of why it wasn't working. Then in the end we got a structure, but we needed many images and many molecules. It had worked and we got a structure and this is a kind of model of the structure. This is made soon after that. It has about five or six side chains and it's got this long molecule in the middle, which is the vitamin A aldehyde.

Probably in 199v0, we realized that although we've got a structure, the amount of signal that you extracted from the image was a tiny proportion of what you ought to. We reckoned we used about five thousand times more data than we really needed. Although it was working, the improvements that came after that made one thousand fold improvement. If you were to try and do this again it would take you not very long, an hour, to do.

So the methods and instruments and all the equipment and computational methods they've all greatly improved now.

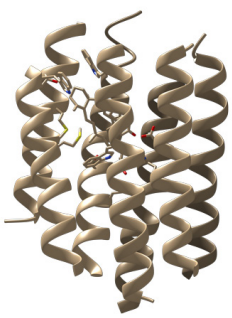

Figure 2 | Bacteriorhodopsin single monomer with retinal molecule between 7 vertical alpha helixes (PDB ID: 1BRD)

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Conflicts of Interest
There are no conflicts to declare.

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