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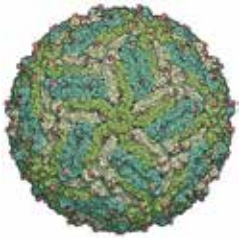
El creciente poder de la CRIOMICROSCOPÍA ELECTRÓNICA

Por RICHARD HENDERSON
Premio Nobel de Química 2017



Durante los últimos años, la criomicroscopía electrónica (crioME) de partículas individuales ha experimentado un salto cuántico en su capacidad de resolver estructuras, debido a la mejora de los microscopios electrónicos, de los detectores de electrones y del software de procesamiento de imagen, lo que ha revolucionado la biología estructural. Utilizando la técnica inventada por Jacques Dubochet y sus colegas, se genera mediante congelación ultrarrápida (utilizando etano líquido) una capa delgada de agua vitrificada en el que las macromoléculas a estudiar (p.ej. proteínas) se disponen en muy distintas orientaciones. El registro de las imágenes de esas partículas y su posterior procesamiento mediante técnicas computacionales sirve para determinar la estructura tridimensional de esas macromoléculas, con frecuencia a resolución casi atómica. En esta conferencia, Richard Henderson describe algunos resultados recientes y las barreras que se pueden sobrepasar en el futuro. La crioME se ha convertido en una poderosa herramienta de la biología estructural, pero todavía hay muchas mejoras que pueden llevarse a cabo para alcanzar sus límites teóricos.

We're still in the infancy of the method. The detectors can be improved. The microscopes can be improved. Everything can be improved. So we're at a very fortunate stage

We have always thought there were four methods for finding out about things in structural biology. Before the experimental methods were powerful enough, what many people did was the model building at the beginning, both for proteins and for nucleic acids. Then, as the methodology developed, X-ray crystallography was the first one to develop enough power to determine the structures. I'll focus on myoglobin and hemoglobin from the work of the two scientists who were founders of structural biology at the MRC Molecular Biology Lab in Cambridge, Max Perutz and John Kendrew.

A second type of model building came from X-ray diffraction patterns. Rosalind Franklin and her small group at Kings College, London, particularly Raymond Gosling, her PhD student, took pictures like the famous photo 51, an experimental evidence. It showed a X-shaped structure with helical parameters, and then a peak at 3.5-Angstrom resolution along the axis. Watson and Crick put that together with lots of other indirect information and came up with another model, which the wife of Crick drew and was published in Nature in 1953. Watson and Crick and Wilkins shared the Nobel Prize for Physiology or Medicine in 1962. Unfortunately, Rosalind, who most of us are sure would have been sharing that prize, had ovarian cancer and died in 1958.

Those were the two most important contributions for model building in proteins and nucleic acids. At this point, the experimental method started to come in. Max Perutz and John Kendrew, under JD Bernal and Lawrence Bragg in Cambridge, started a group to try and work out the structures of proteins. Kendrew was a PhD student at the time, although he was older because he'd been doing technical developments involving evaluating weapons during the wartime, came to work with Perutz and made 25 different types of myoglobin crystal, different species. The sperm whale myoglobin was the best one and they worked on this. Two charcoal sketches were made by Lawrence Bragg, who developed a new way of thinking about X-ray diffraction from crystals, which they called Bragg reflections (Braggs Law in 1912). He and his father shared a Nobel Prize in Physics in 1915. Kendrew and Perutz had a Chemistry Nobel Prize in 1962 for their work on myoglobin and hemoglobin.

They both worked unsuccessfully initially —Perutz for about 30 years, Kendrew for about 15 years—, but eventually solved the problems and got a structure. Perutz wrote a review in 1948 at a meeting in Cambridge where he was describing their great enthusiasm and optimism for solving structures by X-ray crystallography. He explains that, "At first view, an attempt to solve the structure of a protein like hemoglobin looks about as easy or as difficult as a journey to the moon." If you remember, the first man on the moon was in 1968, only 20 years later. So it wasn't so difficult, actually. It turns out that Perutz and his structure of hemoglobin also took 20 years. He said, "It's not only that it looks about as promising, it's exactly as promising". That was the first experimental observation

of the same alpha helices that Linus Pauling had predicted about six or seven years earlier.

They went on without a lot of time and two years later they had another model. They didn't quite know how to represent it, so they persuaded an artist, Irving Geis, to do a watercolor painting where he altered, ever so slightly, so that the atoms were all clear. You could see every single atom in a myoglobin structure, something like 1,000 atoms, so 150 amino acids. In the middle, there was a heme group, an organic structure with an iron atom at the middle. The oxygen binds directly to the iron atom with two histidine residues, a proximal and a distal histidine with the oxygen-binding site. That was the first protein structure.

It took Max a few more months and they got a model of hemoglobin. It was four times bigger than myoglobin with two alpha chains and two beta chains and the same heme group. But it took another 10 years to go from that low-resolution model —two years behind the myoglobin— to a high-resolution model. These were the first two structures, hemoglobin and myoglobin. Then there were some enzymes and tens of other structures.

In the early 1980s, Kurt Wüthrich started to use the indirect methods of chemistry, nuclear magnetic resonance spectroscopy, which had been developed by Richard Ernst in Switzerland. Both Ernst and Wüthrich were given Nobel prizes for their work, Ernst for developing this fully transformed method, and then Wüthrich for applying it to proteins.

Now we come to electron microscopy

Now we come to electron microscopy, often called EM, and cryomicroscopy often called Cryo-EM. There are other types, but the most powerful one at the moment is — rather than taking pictures of crystals, helices

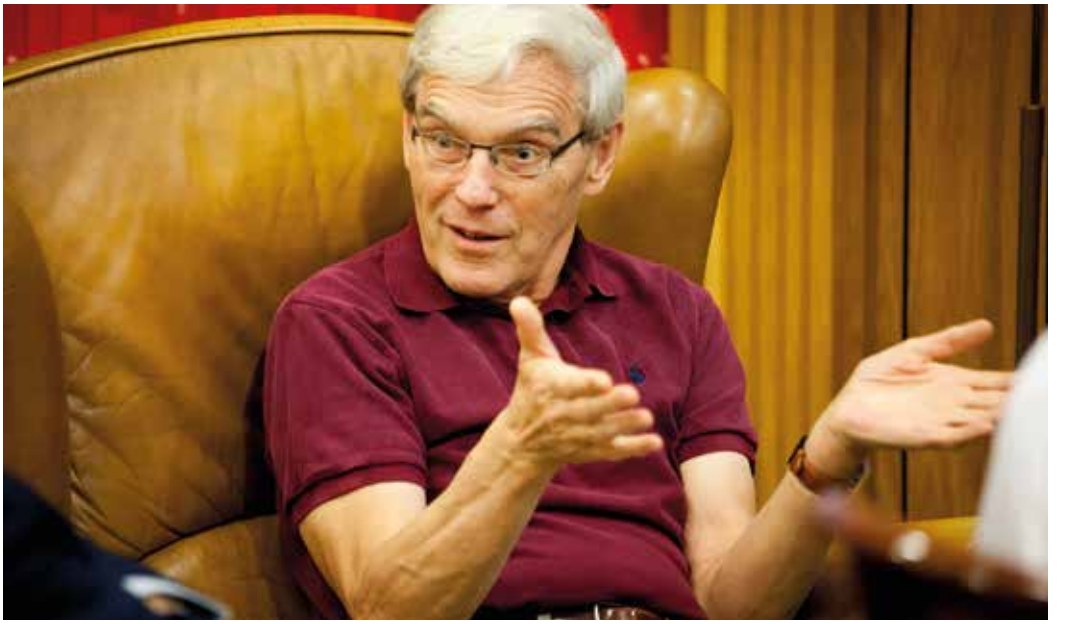
or high-symmetry particles— when you take pictures of single particles. Originally, without cryo, people like Joachim Frank were doing a lot of work with single-particle electron microscopy rather than cryo-electron microscopy. But these three methodologies together have become quite powerful. My own background involved coming into this from X-ray crystallography to electron crystallography. The real power of it came when a method to make vitreous thin films of liquid were developed by Jacques Dubochet and his colleagues, who were then at EMBL in Heidelberg. That's probably the most important development that's really opened up the field. Of course, in order to make it work, the microscopes and the detectors had to be improved.

Coming back to the electron crystallography, I met Nigel Unwin in 1973. I was a protein structural biologist trying to use X-ray crystallography to work on the structure of a protein, which form 2-D crystals in the membrane of a bacterium. We were taking purified membranes, dissolving them in detergent, trying to make 3-D crystals. We got them, but they were very bad 3-D crystals. Actually, from 1975, it took about another 20 years before three-dimensional crystals and bacteria were good enough to determine the X-ray structure. When I met Nigel, he came from material science. He was a metallurgist doing electron microscopy. But he was thinking how to determine the structure of proteins without having heavy metals or anything like that. We worked for a couple of years on these crystals without anything —we sometimes embedded them in glucose or ethylene glycol— and got diffraction pattern. People could think of these of being exactly the same as the X-ray diffraction patterns, but they are not. The diffraction pattern is electrons being diffracted off the crystals rather than x-rays. So we were able to, without cryo, at room temperature get a model at about 7-Angstrom resolution.

It wasn't until we switched our efforts to doing electron microscopy —where you take images, process them in the computer, and find out where the different features are located physically in the structure— that we began to see instead of a smooth feature, features protruding from the seven alpha helical parts of the protein. Eventually, in 1990, we got a map which showed not only the seven transmembrane features, but now protruding from them there are periodic structures that are the side chains of the bigger amino acids. We were able to interpret that in terms of an atomic model in 1990.

But it's Jacques Dubochet who developed the method of plant freezing that has really opened up the field. In 1978, John Kendrew recruited him at EMBL in Heidelberg, specifically with the idea of investigating the behavior of water when you freeze it, and also to develop liquid helium, a very low-temperature microscopy so that you can get images without the water getting in the way of it. These were two of the apparatuses that he developed.

Alastair MacDowell, from Scotland, and Mark Adrian were two postdoctoral researchers working with Jacques. MacDowell developed the idea of taking a little electron microscope grid with droplets of water and plunging it into a little container with liquid ethane in it at slightly above its freezing point surrounded by liquid nitrogen. Alastair McDowall was the one who developed the liquid ethane plunge freezing. Then, Marc Adrian had an idea. Rather than spraying on droplets, you put a big drop on and you blot it with filter paper —nothing could be easier—, and then you plunge it. This was either a pair of forceps with a grid in it falling



under gravity —and by the time it reached the ethane, it would be going about one meter per second—, or sometimes they thought that wasn't fast enough and they would use elastic bands. The two of them together in Jacques' group developed this and published it in 1988.

This is done by starting with a petri dish with three-millimeter electron microscope grids, they have a thin film of carbon with holes in it. Then, you apply your droplet of liquid, two or three microliters, onto one of these grids held in a pair of forceps and blot it with the filter paper —this is the Marc Adrian method. Then you plunge it into liquid ethane —this is Alasdair McDowall's method. That is still the method that's used 30 years later. We all think there must be a better way, but so far, no one has developed one. There is also the official Vitrobot computer-controlled version of Adrian, McDowall and Dubochet's method, and they are, of course, very popular. However, many of us still use the old piece of equipment, which never goes wrong.

The first picture that Dubochet's group took made a lot of impact —a paper Marc Adrian et al. published on the front cover of Nature in 1984. It is a picture of the adenovirus which was the life's work of Lennart Philipson, who was the second director following on from John Kendrew. Dubochet was recruited to work on ice, so he developed the ice to do low-temperature microscopy. Then he applied it to the specimen that was the specimen of the second director of the EMBL. Jacques was very popular at EMBL, but he eventually went to Lausanne in 1987, where now he's retired. It took seven years to go from images like those to computer-processed structures —35-Angstroms resolution—, and that was Phoebe Stewart in 1991. But the resolution wasn't high enough yet.

20 years after, in 2010, Hong Zhou is still taking pictures of adenovirus on film. The microscope has been improved. It's higher voltage, better vacuum, more stable stages, and a very bright field emission gun, thousand times brighter than the electron guns used to take. You can see inside the adenovirus particles a lot more fine detail. Hong Zhou group got images in which you could see the beater sheet structures, the same ones that

Pauling had predicted back in 1951. That was 3.5-Angstrom resolution instead of 35, so 10 times better. That's a linear improvement in resolution, so that's 1000 times in 3 dimensions more data. In the early 2005 to 2010, the methodology was being improved, and there were a lot of these virus structures where you could see maps and densities that were beginning to be at the same kind of resolution you saw with X-ray. But it wasn't until a little bit later that there were further improvements that really opened up the field.

Tim Baker and I wrote a review in the year 2000 where we tried to explain what is cryomicroscopy. It was published in the International Tables for Crystallography Volume F. In this particular journal there were about 88 chapters about different types of X-rays and only 2 about electrons. One was about electron diffraction, written by a man called Wah Chiu, who is now in Stanford. And the other one that Tim Baker and I wrote on electron microscopy. We thought we should try to explain in its breadth what it involves. If you buy the equipment, you can apply it to many different types of specimen.

Bettina Böttcher was a post-doc at the MRC Lab in Cambridge working with Tony Crowther. With a lot of work, they have managed to push it to below 10-Angstrom resolution. That was the very first subnanometer, below 10-Angstrom resolution structure done by a single particle cryo-EM. But in parallel, there were also people working on helical arrangements. That allowed them to understand roughly how muscle contraction was working. And that was Verna Colbrant's work at EMBL following on from Dubochet. From 1990 to 2005, electron crystallography was quite popular because it gave you a higher resolution.

The higher resolution became more possible

without having to have crystals. One of the methodological developments that were very important in this was the development of better detectors than film. One is called direct electron detectors because you fire electrons directly into a sheet of silicon, which has electronics underneath it, and count all the electrons as they arrive. The film had what's called detective quantum efficiency. That means, what proportion of the electrons that are arriving in your detector can you detect as though they were noise free. Of course what we want is 100% of them to be perfectly detected at all resolutions. The new direct electron detectors are Gatan K2, FEI Falcon, and Direct Electron. Three companies made three different detectors all based on silicon. Slightly different in their design, but all of them are better than film. When these detectors came in, suddenly we were getting better images. And also film; it's one piece of film. You expose it and develop it. That's the end of it. When you press the button to take

a picture, these detectors take a sequence of exposures like a movie, all of them with very low noise levels. It has two advantages. First, it's a better detector. Second, you can then do subsequent image processing to remove any blurring or deficiencies in the image. When that was done, you get really nice pictures of very interesting structures that had been intractable by any of the other methods. Now you could just look at the pictures by eye, and you can see more or less what you're going to try and then do it in the computer. Of course, these images encouraged people, and lots of really much more sophisticated computer programs were written afterwards.

Bettina Böttcher and Tony Crowther worked on hepatitis B structure. When she first worked on it, it was on a sample that Nikolay Kiselev brought from Paul Pumpens' group in Latvia. They sent the clone and some protein, and when they first did it with the older microscopes, they had a 30-Angstrom resolution structure, so different symmetries [xT = 3T = 4], icosahedral. You could see the protrusions, but no detail in it. Then, when Bettina worked with a better microscope — field emission guns, but still on film—, she was able to get a sub-nanometer structure with the protrusions where you could see a bundle of four alpha helices. More recently, again the same group about adenovirus, Hung Cho's group, 2013, published a structure of hepatitis B where you could see the protrusions and the alpha helices, they're all very well resolved. You could see side chains, but this was also still taken on film before the new detectors came. More data and better data, and then higher resolution. But now, with the new detectors, you can get better resolution with much fewer data.

I want to move on to relatively recent structures that have really caused this to be a kind of revolution in structural biology. One of them was the work on ribosomes. All of the early atomic structures of ribosomes were done 30S, 50S —the whole ribosome was all done by x-ray crystallography. Three people were given the Nobel Prize in chemistry for determining the structures of the ribosome in 2009. That was before any of these new and more powerful cryo-EM methods came in. Alexey Amunts is one of the key members of cryo-EM facility in Stockholm now. He had been working in Cambridge on bovine mitochondrial ribosomes trying to make crystals. But mitochondria don't have many ribosomes in them and when you try to purify it they're always contaminated 50/50 with cytoplasmic ribosomes. So what they did is simply to take pictures. Alexey was learning with help from Xiaochen Bai in Sjors Scheres' group, so it was a collaboration between a cryo-EM group and a ribosome group. Analyzing pictures, they all looked the same by eye, but in the computer you could sort them out. This is called 3D classification.

Sjors Scheres started that type of analysis, 3D classification, in Madrid using Xmipp, an earlier type of software. One of the earliest 3D classifications were between 2005 and 2007, on the ribosome, but are much lower resolution data. Using the software RELION that Scheres had developed after he moved from Madrid back to Cambridge, they were able to get structures for the component that they were interested in. They could also refine the orientations of the particles either on one of the major components or the other, the large or the small subunit, and then get really good maps of both parts of the structure from the same dataset.

Now there are hundreds of structures being published, all of the structures that were very difficult or impossible by other methods. When people have structures, you deposit your coordinates, and until you have a high resolution, you don't have coordinates, you just have blobs. So this didn't happen. In electron microscopy, there were very few atomic models being deposited 10 years ago, 19 compared to 6,000 from X-ray crystallography. That is a ratio of 300 to 1, 300 times more atomic resolution models being done by X-ray crystallography than done by electron microscopy. By 2015 it was something like 20 times more by X-ray than EM. Of course, it's now continued. But the number per atom X-ray has continued too. Now there are over 10,000 new structures being deposited per year, although many of them are homologous. We thought it was going to plateau but actually it's continued to increase. NMR, which was very popular, peaked in 2007. Now NMR is used for other types of work than *de novo* structure determinations, for example, ligand binding with low-affinity ligands. EM structures had not quite reached, or they had just exceeded it. We need to look forward to know where to put our effort and our money and how many people should work. But it's very difficult to predict the future. We thought what would be good would be to plot the ratio of X-ray versus EM on a long rhythmic scale. It was several hundred to one five or six years ago. When the graph was made, we were not down to about 10 times more worldwide. It looks like in October 2023 we will be on parity. And parity seems pretty good. But Tom Blundell who's another structural biologist

in Cambridge said, "This underestimates the importance of cryo-EM. Although it's true you may reach parity, the structures that you do by cryo-EM are often a lot bigger than the structures that you do by X-ray, and also they're more interesting."

There is an interesting picture published, nine months ago, by a young student called Maryam Khoshouei who was working at the Max Planck Institute in Germany. She said, "I think we should try hemoglobin." This is Max Perutz's structure. Her supervisors rejected the idea, but she just ignored them and collected images —starting on Friday, finishing on Monday— using the faceplate, which many people are using now. She got some images, processed them to the classes and so on, and ended up with a 3-D structure at 3.2-Angstrom resolution. That was remarkable. This is the smallest high-resolution structure that's been determined and it's entirely the initiative of a student who is now working for Novartis in Switzerland. There is another picture that came out four or five months ago and I don't think it's been published properly. The paper was published in Twitter. It turns out that a group at the Salk Institute in the USA got a structure that's at about 1.8-Angstrom resolution. They haven't done anything unique but they read all the papers everyone else has written and they did everything everyone else said correctly. And so they got a really good image.

We're still in the infancy of the method. The detectors can be improved. The microscopes can be improved. Everything can be improved. So we're at a very fortunate stage. It's already working quite well. Lots of people are coming into switching their emphasis in structural biology say from X-ray crystallography to cryo-EM or they're doing both. And we have other future developments to look forward to.

Bio

RICHARD HENDERSON

Es biólogo estructural, con formación en Física por la Universidad de Edimburgo. Después de un doctorado en el Laboratorio de Biología Molecular del Consejo de Investigación Médica (LMB-MRC; Cambridge, Reino Unido) trabajando en mecanismos de enzimas, desarrolló un interés en las proteínas de membrana como investigador postdoctoral en Yale. De vuelta al LMB-MRC y con Nigel Unwin, utilizó la microscopía electrónica para determinar la estructura de la proteína de membrana bacteriorrodopsina en cristales bidimensionales, primero a baja resolución (1975) y más tarde a resolución atómica (1990), la primera proteína resuelta con esta técnica.

Durante los últimos 20 años, ha trabajado en varios aspectos del desarrollo de la metodología de la criomicroscopía electrónica de partículas individuales (crioME), que recientemente ha alcanzado el estado en que es posible obtener estructuras atómicas de una amplia variedad de complejos macromoleculares rutinariamente. Todos estos desarrollos han sido reconocidos por la Academia Nobel, que en 2017 le concedió, junto a Jacques Dubochet y Joachim Frank, el Premio Nobel de Química.

En los últimos años, y junto con Chris Tate, ha desarrollado el método de "termoestabilización conformacional" que permite que cualquier proteína de membrana se vuelva más estable y al mismo tiempo retenga una cierta conformación de interés. Esta técnica ha ayudado a la cristalización y posterior determinación de la estructura de varios receptores acoplados a proteína G (GPCR), que son proteínas de gran interés médico. Estos desarrollos han permitido la creación, en 2007, de la empresa Heptares, que tiene en la actualidad unos 125 empleados.

Ha sido jefe del departamento de Biología Estructural del LMB, subdirector y director. Es miembro de EMBO y FRS, y es poseedor de un gran número de premios y distinciones.