

Molecules – On Ice

Cryo-electron microscopy facilitates the precise imaging of tiny structures, such as molecules, right down to the atomic level. For their contribution to the development of this technology, British molecular biologist Richard Henderson, German-born American researcher Joachim Frank and Swiss biophysicist Jacques Dubochet were awarded the Nobel Prize in Chemistry in 2017.

At the **Max Planck Society's Fritz Haber Institute** in Berlin, former Research Group Leader Friedrich Zemlin was also involved when the method carved out a place for itself in biology in the 1980s.

TEXT **ELKE MAIER**

In order to make inroads into the tiniest dimensions of life, scientists like to put their research objects on ice: they place them on a tiny grid and immerse them at lightning speed in an ethane bath at minus 196 degrees, causing the sample to freeze within a few thousandths of a second. They then use electron beams to X-ray their flash-frozen specimen.

Using this cryo-electron microscopy (*kyros* means “cold” in Greek), they compile thousands of individual images to form one composite image – with spectacular results. Bacteria that attack cells, the most delicate structures on the surface of the Zika virus, and incorrectly folded proteins in the brains of Alzheimer's patients are just some of the images they've obtained. Researchers can even “freeze” molecules in motion to visualize the complicated processes in the cell interior, such as the manufacture of proteins in the ribosomes, the protein factories of the cell.

For a long time, though, no one believed that the electron microscope would ever be suitable for imaging biological objects: to ensure that the electrons are diffracted solely by the specimen,

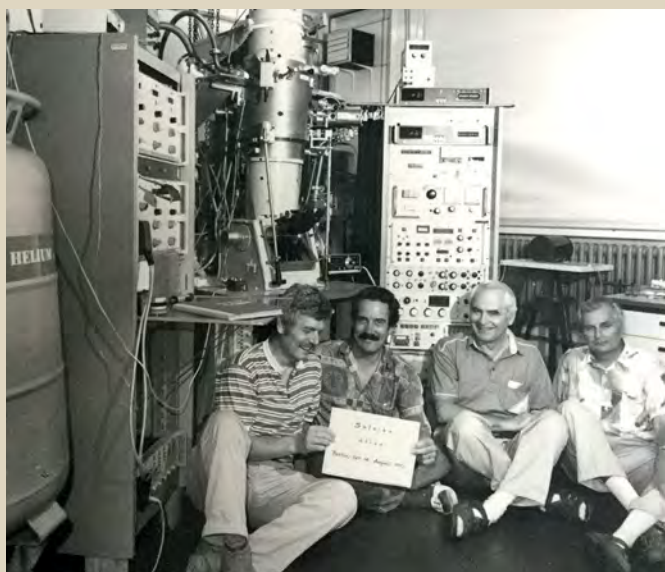
there is a high vacuum in the interior in which hydrated specimens, such as cells, would normally dry out immediately and shrivel up into unrecognizability. On top of this, there is the damaging effect of the radiation: Ernst Ruska, the inventor of the electron microscope, once even caused a metallic filament to evaporate – not a good sign for the far more sensitive biological samples!

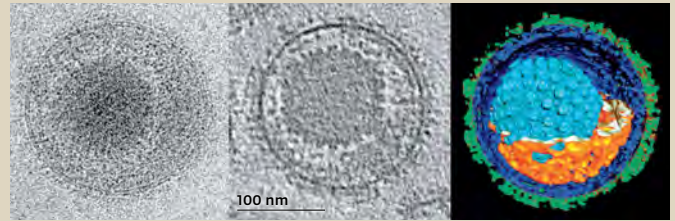
Richard Henderson, too, who carried out research on membrane proteins at the MRC Laboratory of Molecular Biology in Cambridge in the 1970s, initially didn't even consider the electron microscope. Instead, he tried to ascertain the structure of proteins using X-ray crystallography – the method of choice at that time. However, he soon reached the limits of this technology: some proteins couldn't be obtained in sufficient quantity, while others didn't form any crystals at all. That left the electron microscope as the only device capable of displaying the molecules in their natural environment – the cell membrane.

Henderson's favorite protein was bacteriorhodopsin – a red pigment that exists in the cell wall of the halophilic (salt-loving) archaeon *Halobacterium salinarum* and sometimes colors entire salines red. To image the protein in its original state, he and his colleague Nigel Unwin placed the entire membrane in the microscope and selected the lowest radiation dose possible. The trick that helped the researchers was to coat their specimens with a sugar solution, which proved to be effective protection against dehydration. Because the protein had such a regular shape, it was possible to reconstruct its structure in 3-D even with the low-contrast images.

The photo appeared in the journal *NATURE* in 1975 and shows how the molecule's protein chain weaves its way through the cell wall seven times. The resolution is 0.7 nanometers (billionths of a meter) – roughly the length of seven atoms laid side by side. At the time, it was the best depiction of a protein that had ever been achieved with an electron microscope.

Saying goodbye to SULEIKA: Friedrich Zemlin and his team gather one last time in front of the cryo equipment that was used to produce the Nobel Prize-worthy images of bacteriorhodopsin. The famous lens was subsequently removed and installed in a more modern microscope. From left: Friedrich Zemlin, Rolf Meilicke, Erich Beckmann and Klaus Heinrich.





History of a virus: The researchers capture their object from various angles to get a 3-D view of *Herpes simplex*. They combine these 2-D snapshots to form a composite image. Shown here are a microscope image (left), the reconstructed and denoised tomogram (center) and a 3-D reconstruction (right).

But Richard Henderson wasn't yet satisfied. He wanted to show the precise atomic structure of his molecule. In particular, the work that was being done by a team in the Electron Microscopy Department at the Max Planck Society's Fritz Haber Institute (FHI) in Berlin convinced him that there was still room for improvement: in 1984, Elmar Zeitler and Friedrich Zemlin, together with colleagues from the University of Arizona, published the structure of the rattlesnake venom Crotoxin with a resolution of 0.35 nanometers.

"We had a superb microscope at the Institute," says Friedrich Zemlin. "In particular, the helium-cooled, superconducting lens developed at Siemens in Munich by physicist Isolde Dietrich was fantastic. It was part of a laboratory setup that we had put together at the FHI and dubbed SULEIKA, a German acronym for *supraleitende Kryo-Apparatur* – superconducting cryo-equipment." From 1984 on, Richard Henderson frequently traveled to Berlin to work with SULEIKA.

On the other side of the Atlantic, at the New York State Department of Health, Joachim Frank had already been tinkering with image-processing algorithms for several years. The German-born researcher, who later acquired American citizenship,

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It seems that low-temperature microscopy and deep-freezing herald a new chapter in molecular biology: the direct observation of the most elementary vital processes under the microscope.

had been a doctoral student at the Max Planck Institute for Protein and Leather Research in Munich, the forerunner to the Max Planck Institute of Biochemistry in Martinsried. His mentor was Walter Hoppe, an expert in X-ray crystallography who had moved into electron microscopy in the early 1970s.

Hoppe's specialty was the 3-D reconstruction of complex molecular structures, such as enzymes. Using tomographic technology, he imaged one object from various angles and combined the images on the computer to form a three-dimensional composite image. The main problem with this was that the harmful radiation dose increased as a result of the multiple photos.

Frank tried to avoid this by producing a single snapshot of several identical particles in a solution. This reduced the radiation exposure considerably. However, it made analyzing the image all the more complicated, as the particles were jumbled up and crisscrossed and often didn't stand out well from the background. Contamination also occurred, which was a huge challenge, particularly given the lack of computer processing power at the time.

Nevertheless, Frank managed to develop algorithms to search through the widely scattered particles for recurring structures and to form from them a sharp, three-dimensional composite image of the structure. Together with physicist Marin van Heel, who later also worked as a researcher at FHI and who is considered to be a pioneer in the imaging of individual molecules, Frank pub-

lished the first programs in 1981. Frank's SPIDER software package, a comprehensive tool for computer-based image analysis, was published that same year.

One issue remained outstanding: how to protect biological samples from drying out in the vacuum of the electron microscope. Richard Henderson used a sugar solution in his bacteriorhodopsin studies, but that wasn't suitable for all specimens. Some researchers had already tried to freeze their samples, but ice crystals formed in them, which destroyed the delicate structures in the sample and also deflected the electron beam.

That ice really was the way forward was proven by Jacques Dubochet, who was researching precisely this issue at the European Molecular Biology Laboratory (EMBL) in Heidelberg at the time. One idea was to chill the sample so quickly that there was no time for ice crystals to form. Many colleagues considered this to be a utopian dream, as, in order for this to happen, the sample would have to reach a temperature of less than minus 150 degrees Celsius within a few thousandths of a second – a cooling rate of more than 10,000 degrees Celsius per second.

But Dubochet managed to achieve the seemingly impossible. To do this, he cooled water in fractions of a second in an ice-cold ethane bath, freezing the liquid as a glass-like mass – a state researchers call vitrified water. The method proved to be ideally suited to keeping biological specimens from drying out in the vacuum of the electron microscope: vitrified water doesn't form destructive ice crystals, and it allows the electrons to pass unhindered. In addition, the low temperature protects against radiation damage. "Using cryotechnology, we succeeded in imaging paraffin molecules with an electron microscope in the mid-1980s," says Friedrich Zemlin. "That was a real breakthrough, as the long hydrocarbon chains are extremely sensitive to electron beams."

Soon afterwards, the electron microscope became firmly established in biology, as well. Richard Henderson and his colleagues published the first image of a protein at atomic resolution in 1990: after years of painstaking work, the researchers finally succeeded in imaging bacteriorhodopsin at a resolution of 0.35 nanometers. To do this, they combined hundreds of individual snapshots in the computer to form a high-resolution 3-D structure; most of the images came from SULEIKA. Friedrich Zemlin and his colleague Erich Beckmann are two of the co-authors of this pioneering work.

The legendary lens that was used to capture the images was later installed in a more modern Philips microscope and is now located in the Max Planck Institute for Biophysical Chemistry in Göttingen. Institute Director Holger Stark, formerly a doctoral student with Friedrich Zemlin and Marin van Heel at the FHI, had "inherited" the device and taken it with him when he moved to Göttingen in 2001. It was used successfully there for several more years.