Preparing a sample for electron microscopy: The researchers cool a copper grid, measuring just a few millimeters and carrying the protein solution, to nearly minus 200 degrees in liquid ethane within a few microseconds. This prevents the formation of ice crystals in the samples, which would destroy the proteins.

Photo: Frank Vinken

Nano-Sized Lethal Injection

In movies, 3-D effects are spectacular. And also at the **Max Planck Institute of Molecular Physiology** in Dortmund, **Stefan Raunser** finds that three-dimensional images offer a visual feast. His electron microscopes enable him to determine the position of individual atoms with great precision and to study the spatial structure of proteins. In doing so, he occasionally encounters some bizarre constructions.

TEXT **CATARINA PIETSCHMANN**

Nature is full of organisms with unusual life concepts, an impressive example being the tiny nematode *Heterorhabditis bacteriophora*. To reproduce, it crawls through openings in the skin of a beetle larva, where it spits out bacteria that live in its intestine. Bacteria of the species *Photorhabdus luminescens* produce a poison that is lethal for the larva. They then secrete enzymes that break down the dead beetle into its component parts. The bacteria feed on the remains, and the nematodes on the bacteria. Some microbes are not digested, but survive in the worm's intestine and serve as a weapon for the next generation of nematodes.

This is a somewhat unappetizing way of life, but it's useful for the parties involved – a real textbook case of symbiosis. Stefan Raunser finds the bacterial poison and the way it kills the larva fascinating. The poison belongs to the group of Tc toxins, as they are called. Raunser used high-tech electron microscopes to take a very precise look at

this protein complex, which comprises three different proteins, and revealed its structure and mode of operation. Protein A is shaped like a bell with an internal channel. "It has a wide passage and a narrow one, reminiscent of a vuvuzela, the notorious musical instrument favored by South African soccer fans," explains Raunser. Two additional protein molecules, B and C, form a sort of cocoon that envelops the actual poison, a small enzyme at the end of the cocoon protein C. The cocoon binds to protein A at a site intended specifically for this purpose.

As soon as the pH value in the environment decreases or increases, the bell opens up, providing access to the central channel. "The channel is then pushed through the cell membrane like the cannula of an injection needle," says Raunser. A small section of the channel protein produces the energy required for this: it contracts like a metal spring under tension, and the tip of the channel moves forward.

In this process, the cocoon is pulled between the channel and the bell. The cocoon protein C itself cuts off the toxin at its end and injects it through the channel into the beetle cell, with the toxin molecule losing its original structure in the process.

A HIGHLY EFFECTIVE TOXIN

In the cellular fluid, it assumes its regular structure again and changes the arrangement of scaffold proteins, so-called actin filaments. The cell skeleton collapses and the cell dies. "The toxin acts very quickly. The cells collapse within just a few minutes," explains Raunser.

And as if that weren't already crafty enough, the cell even brings in the murder weapon itself. "After the protein complex has docked to the cell membrane, the cell pinches off this piece of membrane inwardly in a small vesicle," explains Raunser. In this bubble-like structure, known as an endosome, the pH value falls, causing the vuvuzela syringe to pierce the membrane of the endosome from inside and inject the toxin into the cellular fluid.

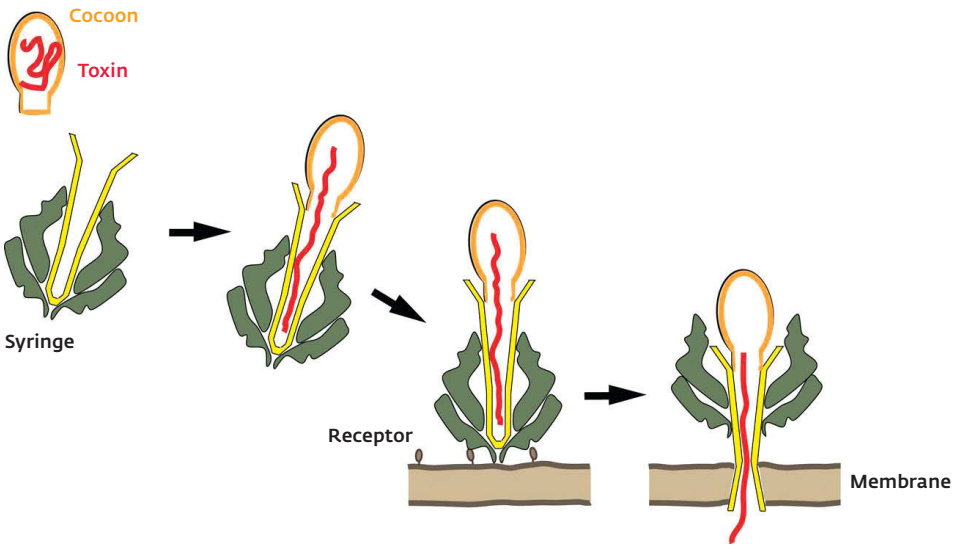
Raunser's team was able to explain the deadly mechanism using cryo-transmission electron microscopy. Unlike with traditional electron microscopy, the sample isn't embedded in a kind of resin, but rather is shock frozen in liquid ethane at minus 196 degrees Celsius. "It occurs so quickly that no ice crystals form that would destroy cells or proteins."

On their path through the sample, the structures of the molecules deflect the microscope's electrons with differing intensities: the greater the atoms' mass, the stronger the interaction. Electron detectors and a computer then produce a two-dimensional image from the captured electrons.

Living cells can't be observed under such conditions, of course. "The vacuum in the microscope would cause biological samples to immediately lose all their water, and the strong electron radiation would quickly damage them," explains Raunser.

For the researchers to be able to reconstruct the spatial structure of the protein complex from the two-dimensional images, they have to take thousands of images from different viewing angles. This means it is advantageous

Photo: Frank Vinken



Left | Yan Nie, Daniel Roderer, Stefan Raunser and Meike Schulte (left to right) analyze the microscope data on the computer. The researchers often don't see the final results of their experiments until months later.

Above | The *Photorhabdus luminescens* Tc toxin, like a classic syringe, is made up of several parts: Two proteins form a cocoon (orange) with the actual toxin (red). A third protein type forms an outer shell (dark green) and a central channel (yellow) that functions as a cannula. If the toxin binds to receptors on the cell membrane and the pH value changes, the cocoon and the channel are pushed forward, and the channel punctures the membrane. Now the toxin is injected into the cell.

when the molecules in the sample are arranged completely randomly: some are lying on their "backs," others on their "stomachs," and yet others are visible from above or below. "What is most important for us are the side views. It's like with photos of people: a person can hardly be identified from the worm's- and the bird's-eye perspective alone," says Raunser. A computer subsequently calculates the three-dimensional structure of the protein using thousands of snapshots.

But it's not just the microscopy itself – the preparation of the samples is also an art of its own. A steady hand and a great deal of skill are needed to load proteins onto a nearly 5-millimeter round copper grid coated with a thin carbon film. Then the whole ensemble must be dipped quickly into the pre-cooled liquid ethane and frozen. "It takes almost a year for new lab members to gain mastery of all the techniques, from preparation to image processing," says Raunser, describing the complex preparation procedure that only a handful of labs in the world master.

Not even a bacterium can survive such a treatment – a beetle larva even less so. Therefore, in order to see how the bacterial toxin develops its lethal effect, the researchers have to employ a trick: they imitate the drop in the pH value that occurs when an endosome is pinched off, and freeze the sample at different points in time. In this way, they can record the various interim states of the toxin and document the individual stages of the intoxication process. The entire cycle can then be played back like a flip-book of individual images.

A TECHNOLOGY CELEBRATES ITS COMEBACK

Thanks to this 3-D cryotechnology, electron microscopy is currently experiencing a renaissance after years of stagnation (see box on p. 50). And just like in its early days, Germany is again one of the leading locations for this technology today. Raunser's microscopes in Dortmund are among the most powerful of their kind worldwide. It's no wonder the 39-year-old from Germany's Palatinate region is serial-publishing the high-profile discoveries made with the methods he refined.

Raunser studied chemistry and biology in Mainz and did his postdoctoral studies at the Max Planck Institute of Biophysics in Frankfurt. He then went to Harvard Medical School in Boston as a postdoctoral researcher before coming to the Max Planck Institute in Dortmund as a Research Group Leader. In 2013, he received more than two million euros from the European Research Council to research this bacterial toxin. Following a brief sojourn at Freie Universität Berlin, he returned to the Max Planck Institute in 2014 and has since headed the Department of Structural Biochemistry.

One of the reasons for the recent triumph of electron microscopy is the incredibly high resolution it allows. "We can almost make individual atoms visible with this technology," says Raunser. In addition, with electron microscopes, it's relatively easy to examine complexes composed of multiple proteins.

High-performance electron microscopes are truly gigantic: the latest gen-

Graphic: Stefan Raunser/MPI of Molecular Physiology

THE HISTORY OF ELECTRON MICROSCOPY

Electron microscopy is a German invention: In 1926, Hans Busch laid the foundation and proved that magnetic coils are similarly good at bundling an electron beam in a vacuum as a lens is at bundling light. In 1932, at the Technische Hochschule Berlin, Ernst Ruska and Bodo von Borries developed an electromagnetic lens. When electricity flows through the coil, a magnetic field is created that can be used to deflect the electron beam. The strength of the magnetic field can be varied by changing the lens current, making the "refractive power" of the lens continuously variable.

From 1937 on, Ruska and von Borries advanced the development of electron microscopes at Siemens & Halske, today's Siemens, in Berlin. Siemens and Zeiss produced the world's first electron microscopes.

This method, which permits 1,000-times higher resolution than light microscopes due to the shorter wavelength of electrons, achieved a scientific breakthrough in the early 1940s. Helmut Ruska, a physician at Berlin's Charité hospital and Ernst Ruska's younger brother, used the technology, also referred to as ultramicroscopy, to visualize such microorganisms as the tobacco mosaic virus, smallpox viruses and bacteriophages for the first time – a real sensation back then.

After the Second World War, Ernst and Helmut Ruska conducted their research in, among other places, the newly founded Max Planck Society. From 1949 to 1974, Ernst Ruska headed the electron microscopy department at the Fritz Haber Institute in Berlin-Dahlem.

In the 1960s, 1970s and 1980s, biologists routinely used the technology to examine cells. Nearly every institute had its own electron microscopy department. These microscopes fundamentally changed our view of life: they made the cell interior visible with previously unparalleled precision.

In 1986, Ernst Ruska was awarded the Nobel Prize – the only one of those involved in the work to receive it, as his brother Helmut and von Borries were already deceased by then.

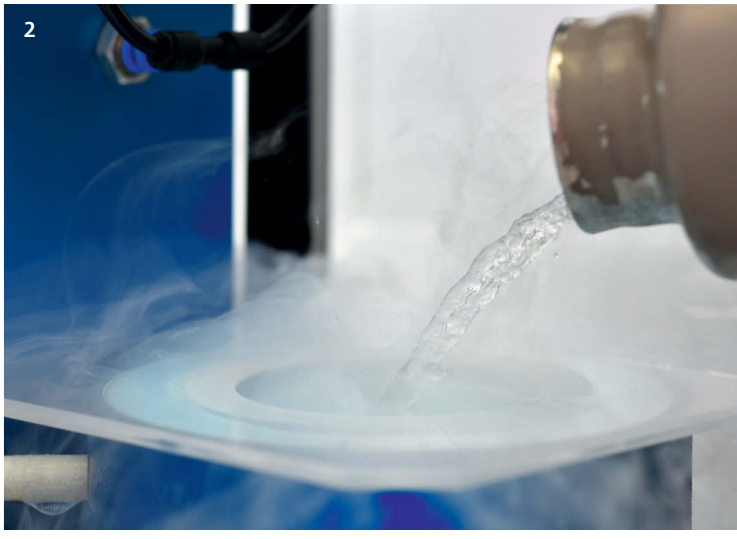
In the 1990s, electron microscopy's importance decreased sharply. New methods entered the competition to revolutionize biology: confocal microscopy and two-photon microscopy. With the help of laser light, science was inching closer and closer to the resolution limit and, unlike with the electron microscope, was now also able to examine living cells. It was now possible to observe many processes live and "in color."

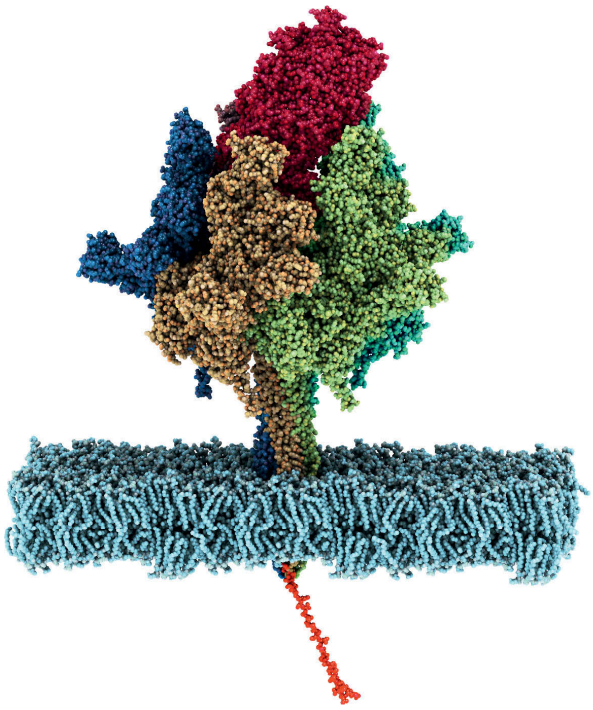
Consequently, many research institutions shut down their electron microscopy departments – only to reopen them shortly after the turn of the millennium: following the sequencing of the human genome, research began to focus on the proteome, the entire set of proteins. Proteins often join together to form larger complexes that then act as molecular machines that perform vital functions, and many of these can only be studied with electron microscopes that afford the required resolution.

Today, Germany is again a global center for electron microscopy. The machines, however, are now manufactured by Japanese and American companies.

For 3-D cryo-electron microscopy, the samples must be shock frozen: Philine Hagel takes liquid nitrogen from a tank (1) and uses it to cool the ethane container (2).

Photos: Frank Vinken (2)





Five molecules of protein A form the injection channel and the outer shell of the toxin complex (four can be seen here). In the interior lies the injection channel through which the toxin (red) is injected into the cell (cell membrane light blue). The cocoon composed of protein B and C is shown in dark red.

eration stands 4 meters high and weighs more than 5 tons. Since they are extremely sensitive to mechanical and electromagnetic vibrations, these instruments have to be housed in specially equipped basements.

ELECTRON MICROSCOPY REQUIRES PATIENCE

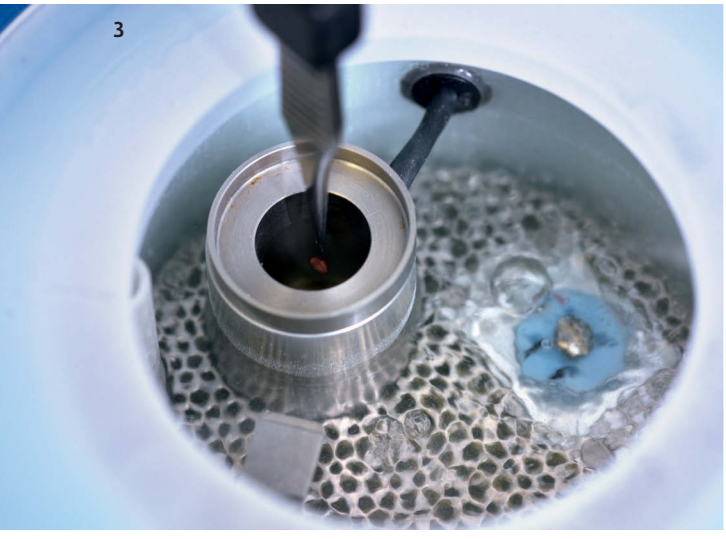
The Max Planck Institute of Molecular Physiology has two electron microscopes for standard tasks. "We use these to look through our samples and choose the best ones." The scientists then examine these using one of the two high-performance cryo-electron microscopes.

The requisite computer capacity alone takes up an entire room. Numerous ventilators are needed to cool the electronic brain as it processes thousands of records simultaneously. It's a long, drawn-out process – a doctoral dissertation in Raunser's group isn't for the impatient at heart. "The microscopy alone takes three months, and the image processing often requires another 18 months. Then it's back to the microscope again," says Raunser.

In clarifying protein structures, the scientist also constantly aims to advance medicine: he hopes his efforts will provide starting points for new drugs. For instance, he is studying the interaction between actin and myosin – proteins that play a role in cardiomyopathies, or diseases of the heart muscle – in muscle cells. Furthermore, he is interested in how the body regulates cholesterol. Three proteins measure whether there is sufficient cholesterol

Photos: Frank Vinken (2), graphic: Stefan Raunser/MPI of Molecular Physiology

The sample is plunged into this container (3) and then put into the electron microscope – here by Oliver Hofnagel (4).



1 | Analyzing the images from the electron microscope requires immense computing power. Alexander Fieroch thus regularly checks the work done by the computer cluster.

2 | Clarifying the mode of operation of these molecular machines in the cell: Stefan Raunser at the Max Planck Institute in Dortmund.

in the body. If not, they ramp up production in the cell. Raunser wants to find out how these proteins cooperate.

EFFECTIVE ATTACK ON BACTERIA

The bacterial toxin produced by *Photorhabdus luminescens* could also be medically important, even though at first glance it doesn't seem to be. Nature also provided microorganisms with toxins that are dangerous to humans, such as *Salmonella* and the pathogen that causes pneumonic and bubonic plague, *Yersinia pestis*.

The findings Raunser's team obtains will help foster a better understanding of how also bacteria such as these operate. Moreover, the Tc toxins could also be used in medicine, as nanoinjectors. This would make it possible to inject drugs into somatic cells – also allowing, for instance, targeted attacks on cancer cells.

"We are currently searching for the toxin receptor on the cell membrane. When we find it and understand how it binds to the cell surface, we aim to alter this region of the protein in such a way that it recognizes cancer cells. This would then make it possible to inject a killer enzyme exclusively into tumor cells," explains Raunser.

He and his team are also trying to insert other active substances into the protein cocoon – using a bacterium's toxin "quiver" as an ultrasensitive drug transporter. But the cocoon could also be used to smuggle in repair enzymes to heal diseased cells.

Incidentally, gardeners – with no awareness of this clever lethal injection – have long taken advantage of the symbiosis between *Photorhabdus luminescens* and nematodes as a natural insecticide. They put a little packet of it into their watering cans, and the toxin of their symbionts unfailingly destroys the insatiable weevil larvae, garden chafers and June bugs that love to feast on the roots of plants. ◂

TO THE POINT

- **The resolution of electron microscopes today is so high that researchers can use them to reveal the spatial structure of proteins. This is ideal particularly for examining proteins that assemble to form large complexes.**
- **Protein complexes work like molecular machines in the cell. Tc toxins from bacteria, for instance, form complicated injection devices with which the microbes can inject a toxic enzyme into cells.**

GLOSSARY

Endosomes: Tiny membrane vesicles that are closed off inwardly by the cell membrane, enabling the cell to transport proteins from its outer shell into the interior. They are part of a transport system comprising a variety of vesicles. On one of these transport pathways, the endosomes fuse with so-called lysosomes, which digest the contents of the vesicles.

X-ray diffraction analysis: A method that can likewise be used to examine the structure of proteins. The X-rays are diffracted by proteins, creating diffraction patterns from which the spatial structure of the proteins can then be calculated. However, this requires that the proteins be in crystal form, which is often technically difficult to achieve. It is very difficult to crystallize large protein complexes.

Photos: Frank Vinken (2)