Diffract, then destroy

A new implementation of X-ray diffraction using free-electron lasers can take snapshots of biological molecules that are inaccessible via X-ray crystallography. As **Philip Ball** reports, the technique can even be used to create stop-motion films of dynamic molecular processes

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When the German physicist Max von Laue realized in 1912 that X-rays could be used to peer into the microscopic world of atoms and molecules, he paved the way for arguably the greatest contribution of physics to biology. Within decades, X-ray crystallography was providing a glimpse at the atomic-scale structure of biological molecules, and by the early 1960s it had become possible to determine the structures of proteins such as haemoglobin this way. And when X-ray crystallography measurements made by Rosalind Franklin and Maurice Wilkins allowed James Watson and Francis Crick to deduce the molecular structure of DNA in 1953, the new era of genetics began.

Now the stage seems set for X-rays to take biomolecular structure determination to a new level. Thanks to the advent of free-electron lasers – sources of very bright, coherent X-ray beams – over the past decade or so, it is becoming possible not only to study complex molecules previously inaccessible to crystallography, but also to move beyond a simplistic view in which the function of biomolecules is determined by their static structure. Instead, we can now see how their role depends on changes in that shape.

At the end of this year, or in early 2017, the world's brightest X-ray free-electron laser, called simply the European XFEL (EU-XFEL), situated in Hamburg, Germany, will take the first steps towards producing its X-ray beams. The facility, costing €1.2bn and based at the DESY (Deutsches Elektronen-Synchrotron) facility, should be ready for its first users by the summer of 2017.

It's not just molecular biologists who are licking their lips in anticipation of what the EU-XFEL will reveal, but scientists from many fields, including materials scientists and chemists interested in surface science, catalysis and reaction dynamics. "The European XFEL will be a unique facility worldwide for years to come," says physicist Marc Messerschmidt, currently a guest scientist at the Hamburg campus.

The challenge is to make an X-ray beam bright enough to elicit a complete diffraction pattern in a single shot from just one molecule

No crystals needed

As the name indicates, X-ray crystallography is traditionally all about crystals. Von Laue realized that beams of X-rays bouncing off the regular arrays of atoms in a crystal would interfere with one another to produce a pattern of light and dark spots or bands. This phenomenon is now called Bragg diffraction after the father-and-son team William and Lawrence Bragg. They showed in 1913 how these diffraction patterns could be mathematically decoded to figure out the distances between atomic planes, and thus the atomic structure of the crystal.

It gradually became possible to decode more complex diffraction patterns, to the point where the structures of molecules such as proteins with thousands of atoms can be deduced. The more complete the diffraction pattern – if it is recorded with strong contrast out to high scattering angles of the deflected X-rays – the finer the resolution of the structure. But because the Braggs' method relies on interference between regularly spaced atoms, it only works for crystalline samples. That's a problem for structural biology, because many important proteins can't be crystallized. What's more, a protein structure in a crystal is a static thing, whereas proteins in cells can be quite flexible and need to deform to do their job (usually, catalysing a biochemical reaction). So a crystal structure doesn't always reveal a molecular mechanism, and sometimes the structure can't be obtained in any case.

But contrary to common belief, diffraction doesn't in fact depend on crystallinity at all. Any photon bouncing off a part of a molecule can in principle interfere with one bouncing off another part – and this interference contains information about the structural relationship between the scattering sites. So if it were possible to scatter a whole lot of X-ray photons at the same time from a single molecule, and record the pattern they make, analysing that pattern could reveal the molecule's structure. In other words, it is possible to do diffraction on single molecules.

The challenge, though, is to make an X-ray beam bright enough to elicit a complete diffraction pattern in a single shot from just one molecule. This is what free-electron lasers make possible. They are an evolution of ring-shaped synchrotron X-ray sources, which exploit the fact that very rapidly moving charged particles such as electrons, travelling in the ring, emit elec-

SLAC National Accelerator Laboratory

Installation
SLAC technician Miguel Pinillas attaches part of a detector at the Linac Coherent Light Source XFEL in California.

tromagnetic radiation when they change direction.

Free-electron X-ray lasers, meanwhile, use the same principle to produce even brighter beams. They typically use electrons accelerated in linear rather than circular channels. Once the electrons are travelling close to the speed of light, they are sent past arrays of magnets, called undulators, which bend the paths into wiggling slalom tracks that generate synchrotron X-rays. The electrons are produced in pulses, and so the X-rays are too.

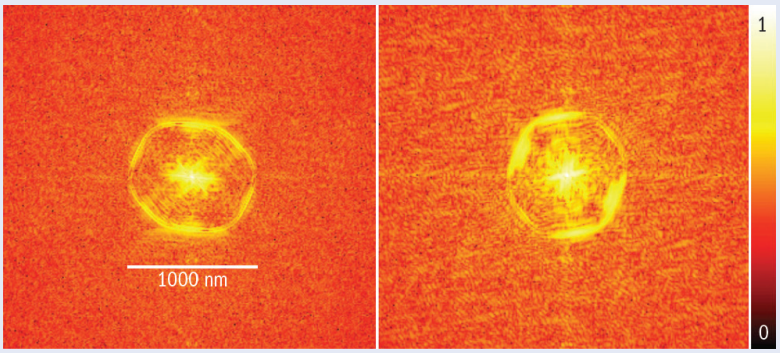
Since 2009 the Linac Coherent Light Source (LCLS) XFEL has been operating at the SLAC National Accelerator Laboratory in Stanford, California, built partly from the old SLAC linear collider used for high-energy physics. Another XFEL, given the acronym SACLA, runs at the SPring-8 synchrotron facility in Hyogo prefecture, Japan, and other X-ray laser sources are about to start up in Switzerland and South Korea. The Hamburg DESY site has been running a prototype XFEL, called FLASH, since 2004.

As well as being brighter than any of these rival facilities, EU-XFEL has the advantage that its pulses will be much, much shorter and more rapidly repeating than any previously available. They will last just a few femtoseconds each, coming at a rate of 27000 times a second, compared with just 120 per second at the LCLS. This means that the EU-XFEL will be able to collect data on molecular structures much faster than is currently possible.

Overcoming damage

A single X-ray photon can wreak havoc with a molecule that absorbs it – which is precisely why X-rays

1 Virus particles come into focus



Seibert *et al Nature* **470** 78

These images are the result of applying autocorrelation – a mathematical tool for finding repeating patterns – to diffraction patterns of individual virus particles captured in two different orientations.

are so dangerous, capable of inducing carcinogenic mutations in DNA. So hitting a single molecule with an intense pulse of such photons will destroy it completely. This kind of beam-induced damage has previously hindered the study of biomolecules, which are often rather delicate: electron beams used for electron microscopy, for example, will tend to fry living cells.

The problem of damage looked very discouraging in the 1990s to researchers wondering if it would be possible to make X-ray microscopes, which in principle should reach down to atomic resolution because of the short wavelength of X-rays. But in 1995 Australian physicist Henry Chapman, working with David Sayre at the State University of New York, wondered if a sufficiently brief pulse of X-rays might produce an image before radiation damage sets in. There were other problems with X-ray microscopes, however, especially the difficulty of developing optics for focusing the beams.

In 2000 Hungarian scientist Janos Hajdu and co-workers at Uppsala University in Sweden showed not only that the pulsed idea could work, but that the challenges of X-ray optics could be bypassed by instead using the bright beams promised from XFELs to record a diffraction pattern. Yes the X-rays will obliterate the molecules, Hajdu agreed: once the X-rays kick electrons out of the molecule's atoms, making them positively charged ions, the repulsive force between the ions compels the molecule to shatter into fragments. But this process only really begins about 10 fs after the initial encounter, whereas Hajdu and colleagues showed that enough X-rays could be scattered within just the first few femtoseconds to permit a diffraction pattern to be recorded (*Nature* **406** 752). The work "essentially gave a roadmap for breaking free of crystals for structure determination" says Chapman.

Encouraged by that idea, Chapman began to attempt experiments. In 2006 he, Hajdu and their co-workers used FLASH in Hamburg to obtain a diffraction pattern from a pulse of X-rays scattered from a silicon nitride film engraved with a non-periodic nanoscale structure – two stick figures and a stick-Sun – before the X-ray beam vaporized it

If the scattering in different orientations is bright enough it should be possible to use mathematical tools to spot patterns and add them up

(*Nature Phys.* **2** 839). They were able to successfully reconstruct the image from the diffraction data.

Strengthening the signal

Imaging molecules was a challenge of another order. For one thing, the scattering from each molecule will be quite weak, unlike the bright spots of Bragg diffraction from crystal planes. But that problem could be overcome in principle by adding up the diffraction data from many molecules, boosting the signal-to-noise ratio. If the molecules have random alignments relative to one another, their scattering patterns too will be in different orientations. If these patterns are bright enough, though, it should be possible to use mathematical tools to spot patterns that happen to have the same alignment and add them up to obtain a good signal-to-noise ratio.

Physicist and microscopist John Spence of Arizona State University suggested another option: using lasers to align the molecules so that they all have the same orientation in the first place. Then there's no need for fancy mathematical tricks before adding up their diffraction patterns. Jochen Küpper and colleagues at the Centre for Free Electron Laser Science (CFEL) – now the hub of these efforts at the DESY site in Hamburg – showed in 2014 that this is possible for small organic molecules with an electrical dipole, which creates a "handle" for lining them up. Küpper and colleagues aligned molecules of diiodobenzonitrile along the axis of its two iodine molecules using the electric field of an intense laser. They sent this aligned molecular jet through the X-ray beam of the LCLS at Stanford and were able to deduce from the diffraction pattern the distance between the iodine atoms (*Phys. Rev. Lett.* **112** 083002).

The scattered signal is stronger if it comes from several molecules at once. Some proteins might form very small crystals – perhaps a micron or so across – even if they won't crystallize into larger ones, and so these microcrystals can be sprayed into the X-ray beam in a narrow liquid jet squirted through a nozzle. But even if such orderly arrays aren't possible, the molecules can be formed into small clusters to elicit a stronger scattering signal.

Molecular movies

In 2011 Chapman and Spence, working with Hajdu along with biophysicists Ilme Schlichting at the Max Planck Institute for Medical Research in Heidelberg,

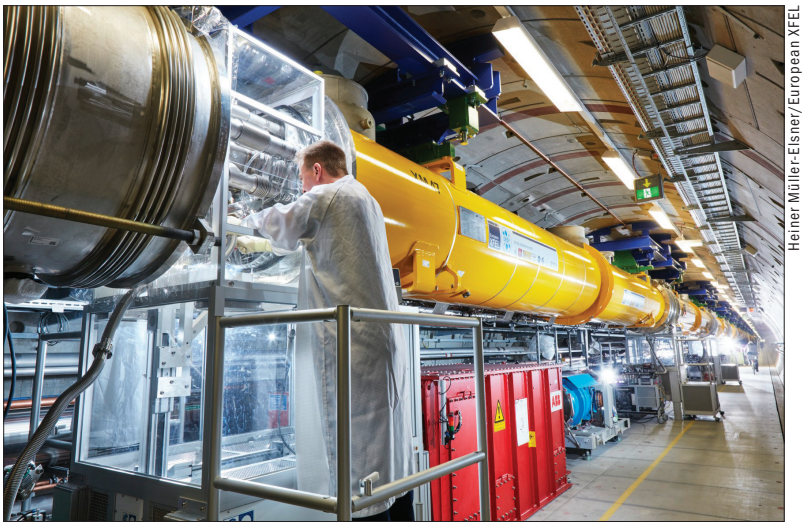
Petra Fromme at Arizona State University, and a host of others, reported two landmark results. Using femtosecond pulses from the LCLS, they imaged single virus particles, and nanoscale crystals of the photosystem I membrane protein – part of the photosynthetic machinery of green plants and algae. The viral images (figure 1) were somewhat fuzzy, with a spatial resolution of just 32 nm, but showed the densely packed material inside the viral coat – and no sign of damage from the X-ray beam (*Nature* **470** 78). And for photosystem I the researchers could merge more than three million diffraction patterns from individual nanocrystals to obtain a structure with 8.5 Å resolution, and with minimal beam damage (*Nature* **470** 73).

Now other biologists were persuaded that there was something in this business after all. "I remember I was teaching a biocatalysis class the day the photosystem I paper came out," says Arwen Pearson, a protein biochemist previously at the University of Leeds in the UK who moved to the Hamburg CFEL this year. "I tore up my planned lecture for the day and raved at a class of fairly bemused chemists about how amazing this result was."

For Pearson the attraction of the rapid pulses isn't simply to allow diffraction to outrun destruction. It means that one can take structural snapshots at different stages of a chemical process, and so produce a kind of stop-motion film that reveals how it happens – how, for example, a protein changes shape as it binds its target molecule (ligand). This idea has been developed in particular by Schlichting at Heidelberg. Last September she and her co-workers used femtosecond pulses from the LCLS to watch the ultrafast dynamical rearrangements of the protein structure of the oxygen-storage protein myoglobin, following cleavage and dissociation of carbon monoxide from its oxygen-binding site. This chemical process sends collective ripples through the molecule (*Science Rep.* 10.1126/science.aac5492).

"Dynamics in structural biology is having a bit of a resurgence," says Pearson. "There's a growing recognition that just one structure isn't enough to really understand the mechanism." This dynamical view of ligand binding, for instance, might be crucial to designing a drug that can bind to a protein and inhibit its function – the old picture of a drug molecule simply fitting like a rigid key into a rigid lock is no longer apt. "This view is not just driven by a few key questions, but rather by the general view that everything in biology is dynamic," says Messerschmidt. "All of the molecular machinery is constantly changing, proteins are continuously being produced, ion flows are controlled through membranes, and much more."

The challenge for time-resolved diffraction, though, is how to set the clock ticking. If you want to follow a process step by step by looking at it in different molecules at different stages, you need to be able to precisely control the timing of the initiation of the reaction relative to the arrival of the X-ray pulse. Pearson is aiming to do this by using clever organic chemistry to build controllable switches into proteins: for example, attaching some chemical group that blocks the binding site until it is snipped off by a pulse of light. Even then, this "release" step has to happen fast compared with the timescale of the subsequent reaction; otherwise it's like trying to time a 100 m sprint without knowing quite when the runners left the block.



Heiner Müller-Elsner/European XFEL

Finishing touches
Technicians make connections between two accelerator modules in the European XFEL tunnel, using a small box as a clean area.

A common language

This work is about as interdisciplinary as you can imagine: using the hardware of high-energy physics and advanced optics, theories of physical chemistry, and methods of chemical synthesis to tackle biological problems. It's not always easy for teams to communicate. "The biggest challenge is the language barrier," says Pearson. "Sometimes something as simple as 'small' has different meanings. I had a lovely discussion with some spectroscopists about an experiment, and it was all going swimmingly until one of them innocently asked 'how small is small?'. I answered that 200–300 µm was our average crystal size. There was a silence. Eventually I ventured to ask what kind of things they regarded as small, and they said 'a few centimetres'."

But centres like Stanford's LCLS and Hamburg's CFEL are slowly forging a common language. Everyone agrees that once the EU-XFEL is up and running, the results should be dramatic. "There's a lot of excitement about the EU-XFEL being finished," says Pearson. "It has three things going for it: brighter pulses, the increased number of pulses, and the ability to do multiple simultaneous experiments." In particular, there is hope that it might finally produce beams bright enough for single-molecule imaging. "The EU-XFEL will allow for the first time to collect molecular movies over the full range of important time scales in a contiguous fashion," says Messerschmidt.

All this won't render conventional X-ray crystallography obsolete. But it should mean that the "crystal" part of the technique becomes optional, and new frontiers in the molecular world will be opened up. Schlichting recalls Lawrence Bragg's words from the early days of crystallography: "It was a wonderful time – like discovering a new goldfield where nuggets could be picked up on the ground." XFELs are providing a very similar spirit of adventure and discovery to that of the Braggs. ■