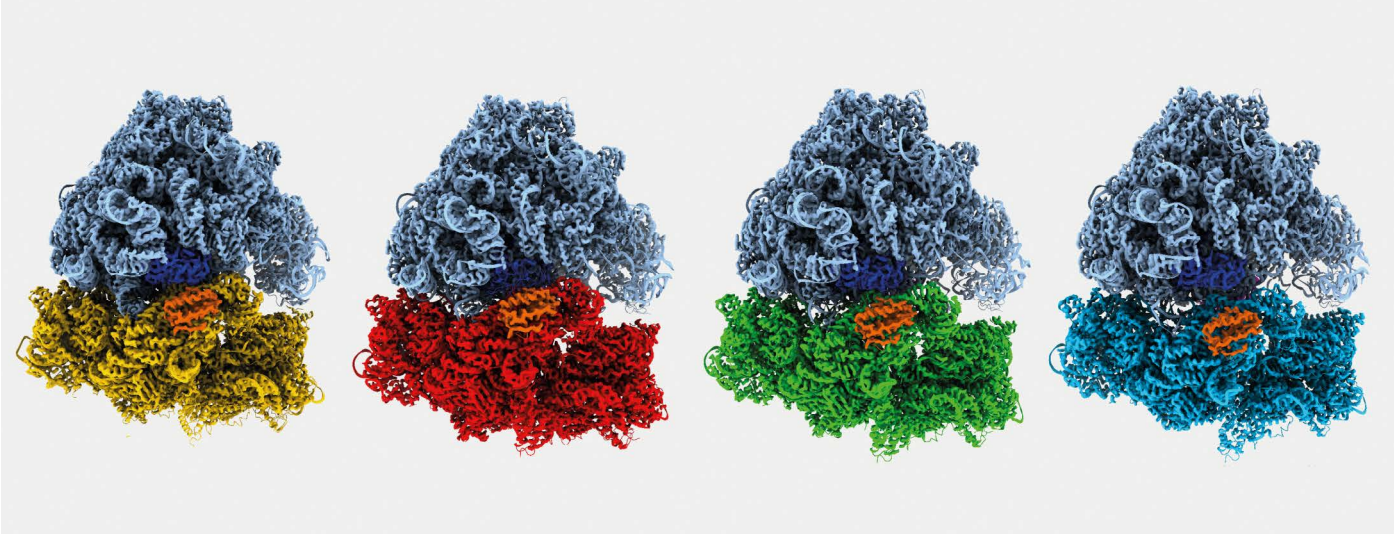


REF. 3

Separation of a bacterial ribosome captured by time-resolved cryo-electron microscopy.

FREEZE FRAME: CRACKING MOLECULAR MOTION

Time-resolved cryo-electron microscopy can capture rapid protein movements, but the need for specialized tools and skills limits the method's reach. **By Michael Eisenstein**

'Blink and you'll miss it' is an understatement in the world of protein biology. A blink lasts hundreds of milliseconds. Proteins operate on a much shorter timescale – on the order of a millisecond, or even faster. "To capture many of the domain motions of proteins that are linked to function, you would have to be able to go down to a microsecond timescale," says Ulrich Lorenz, a chemist at the Swiss Federal Institute of Technology in Lausanne.

Working on that timescale has long stymied biologists, but the rapid evolution of time-resolved cryo-electron microscopy (TR cryo-EM) over the past several years has made it possible to reconstruct dynamic processes with near-atomic detail.

Beyond fundamental biological insights, a deeper understanding of protein dynamics could guide the development of more effective medicines and boost protein-engineering efforts. "Time-resolved cryo-EM could be the natural choice – or only option – to capture [protein] conformations that could be potentially better targeted by drugs," says Youdong Mao, a physicist at Peking University in Beijing, China.

But the technique is niche: these methods require considerable expertise and specialized equipment, and researchers are still working out how to interpret the resulting data. "Nature is under no obligation to be simple," cautions Radoslav Enchev, a biochemist at the Francis Crick Institute in London. And until more laboratories get the tools needed to perform TR cryo-EM, progress in this exciting field will continue to be sporadic.

From stills to cinematography

The basics of cryo-EM are straightforward: researchers apply protein solutions to a 'grid' substrate and flash-freeze them into a glassy structure known as vitreous ice. Using a transmission electron microscope, they then image individual molecules in many orientations – imagine chunks of fruit embedded in jelly. Given enough examples, these 2D 'projections' can be reconstructed into a high-resolution 3D structure.

> **"There were a lot of questions we just couldn't answer with static structures."**

Skilled users can produce structures sharp enough to resolve individual hydrogen atoms. But most experiments image just a single, relatively stable conformation. Structural biologist Stephen Muench at the University of Leeds, UK, struggled for years with the limitations of his cryo-EM studies of a protein known as vacuolar ATPase, a molecular motor that spins as it pumps protons across lipid membranes. "There were a lot of questions we just couldn't answer with static structures," says Muench.

More generally, says Lorenz, biologists have had to choose whether to achieve high resolution in time or in space. Live-cell 'super-resolution' experiments can visualize single proteins at millisecond timescales but cannot discern detailed structural features. Nuclear magnetic resonance can record relatively fast transitions between protein conformations with high resolution, but is limited to small targets. And although time-resolved X-ray crystallography can distinguish both atomic detail and incredibly rapid, femtosecond-scale movement, it does so in a highly constrained setting. "Protein dynamics don't naturally occur in a crystal," explains Lorenz.

In principle, cryo-EM provides an excellent fit for time-resolved experiments. Given enough particles, researchers could theoretically reconstruct every conformational state that a protein can assume. The trick is

to get these dynamic processes to unfold in lockstep, such that the 3D reconstructions can be assembled into a molecular movie. This can be achieved by taking a sample of protein at equilibrium, introducing a ligand or other molecular trigger that initiates movement, and then rapidly freezing the sample at various times after the reaction starts.

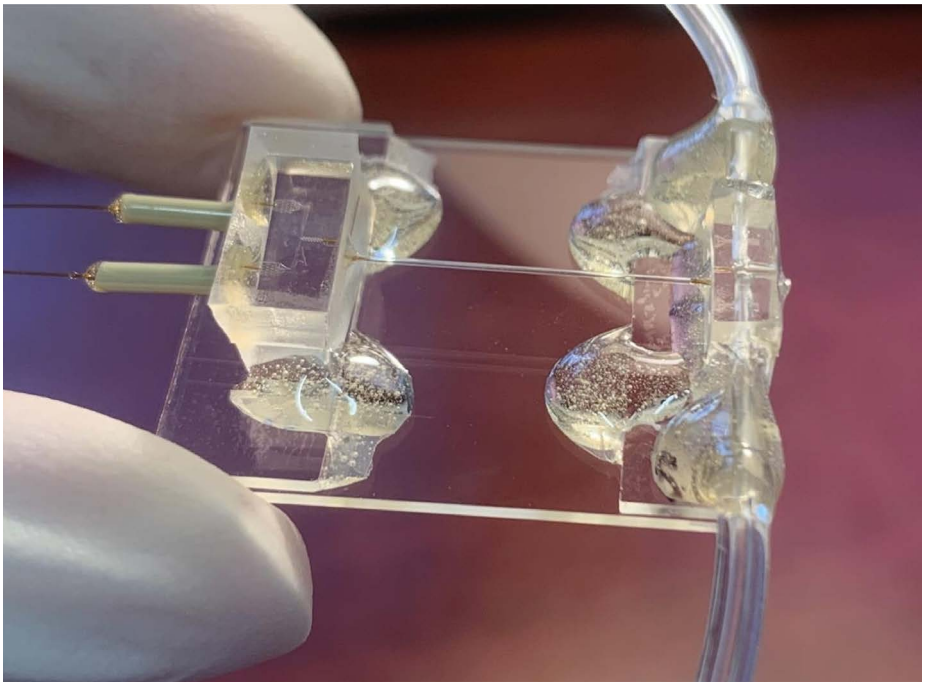
In 1995, for instance, Nigel Unwin, a structural biologist at the MRC Laboratory of Molecular Biology in Cambridge, UK, prepared a grid with the receptor for the neurotransmitter acetylcholine and sprayed it with a solution containing acetylcholine[1]. By quickly freezing the mixture, Unwin captured changes that occurred in the receptor within five milliseconds of acetylcholine binding. Cryo-EM spatial resolution was poor in those days, but other groups have subsequently leveraged similar 'spray-and-mix' approaches to produce detailed dynamic reconstructions with modern, high-resolution instruments.

However, spray-and-mix methods can also introduce considerable uncertainty owing to inefficient mixing between protein and ligand. "Diffusion is happening, so nothing is really under control," says Joachim Frank, a biophysicist at Columbia University in New York City, who shared the 2017 Nobel Prize in Chemistry for his pivotal role in advancing cryo-EM for structural biology. "We wanted to develop a method in which we have control of everything."

As an alternative, Frank's group began working on polymer-based microfluidic chips containing tiny channels, within which a protein and its ligand can be precisely mixed before being sprayed onto a grid and frozen. They described their first-generation instrument in 2015 (ref. 2), and several other groups have subsequently devised alternative 'mix-and-spray' systems.

Contemporary chips incorporate precisely engineered, zigzagging channels for rapid, efficient sample mixing and smooth fluid flow, and mix-and-spray has become the method of choice for many TR cryo-EM practitioners. Last year, for example, Frank and his colleagues used their latest-generation microfluidic design to study how ribosomes are recycled after protein synthesis to begin translation of a new polypeptide chain[3]. "We can see how the ribosome is opened up like a clam-shell upon the interaction with a particular molecule that pries the whole thing open," says Frank. In April, Muench and his collaborators employed their own mix-and-spray system to study actomyosin complexes from muscle fibres, revealing crucial details about how the myosin protein stores and subsequently releases the energy that powers muscle contraction over the course of 120 milliseconds[4].

Still, there's plenty of room for improvement. For one thing, microfluidics-based sample preparation requires a lot of material. "In freezing one time point, you consume several microlitres of sample, which normally is all the protein you can purify over several months," says Mao. His team pursued an alternative approach to study the protein-degrading proteasome complex, using reaction conditions that slowed proteasome activity to a crawl so that time-resolved data could be collected using more conventional cryo-EM methods.



A microfluidic chip comprising three parts: a micromixer, a microreactor and a microsprayer.

REF. 3

An evolving landscape

Researchers led by Rouslan Efremov, a structural biologist at the VIB-VUB Center for Structural Biology in Brussels, have devised a mix-and-spray system that reduces sample-size requirements by an order of magnitude. Their microfluidic device mixes a protein and a ligand in tiny droplets, allowing them to rapidly react in a confined space before they are launched onto the grid using a laser-based mechanism[5]. "The main advantage is that this consumes only about 100 nanolitres per grid," says Efremov, who has used this approach to study complexes that are responsible for protein folding and bacterial metabolism.

Faster frame rates are also in demand. Existing microfluidic approaches have a 'speed limit' of around 5 milliseconds, and although many conformational changes occur in this time frame, others do not. Furthermore, millisecond-scale resolution preferentially captures major intermediates and endpoints for a protein conformational change but misses shorter-lived transition states that are essential for understanding the events leading up to that change.

Lorenz's group took a gamble in its bid to break the millisecond barrier[6]. First, the researchers freeze their protein sample on a grid in the presence of an inactive, chemically 'caged' ligand or activator. The frozen sample is then illuminated with a laser to release the activator compound, after which a second beam is used to quickly melt the ice. After an appropriate interval, the sample is rapidly refrozen, capturing conformational changes occurring in as few as 5 microseconds.

Remarkably, this melting and revitrification strategy does not seem to negatively affect proteins, and Lorenz notes that it can even improve the resolution of the 3D reconstruction by knocking the protein particles into a wider range of orientations. His team is currently using this method to study a variety of viruses and investigate how rapid conformational changes by viral proteins aid host-cell infection. And even faster snapshots might be in reach. "I think 10 nanoseconds or so should be easily possible," says Lorenz, adding that such resolution could capture ultra-fast events, such as the folding of protein structural domains.

When it comes to TR cryo-EM, even minor details matter. For example, the ice layer on the grid needs to be thick enough to protect the proteins from air, which can damage molecules and render them uninterpretable, but also thin enough to maximize image contrast. In a preprint published in April, Enchev and his colleagues presented a platform called Chronobot to tackle this problem[7]. Here, samples are mixed in a reusable glass microfluidic chip and then sprayed onto the grid, which is imaged with a high-speed camera immediately before freezing. A deep-learning algorithm then assesses the ice quality in each

sector of the grid while also optimizing future sample-preparation conditions on the basis of those results. This helps the team to avoid wasting valuable instrument time on bad samples. "This rapid iteration away from the expensive microscope allows us to make sure that we would get a high-resolution data set of any grid that passes this quality control," says Enchev.

Getting things in order

The challenges continue at the end of the experiment, too. Cryo-EM experiments require many thousands of particle images to produce a high-quality reconstruction, and that number increases further when imaging multiple structural intermediates. For one study of the proteasome, Mao says that his group collected nearly 200,000 electron micrographs, each containing hundreds or thousands of individual protein particles. "When we do the data processing, we basically harvest most of the supercomputing power at Peking University," says Mao.

There are also issues in interpretation. Although time-resolved experiments are designed to document conformational states at defined time points after the start of a reaction, the results can be messy — like a race in which each of the runners leaves the starting line at a slightly different time. Many nanoscale forces and environmental factors can subtly influence biochemical processes, but experimental design also plays a part — for example, irregularities in fluid flow in a microfluidic device can 'blur' time points by delaying the arrival of some particles at the grid.

This means that the results at each time point are typically made up of a heterogeneous mix of conformations from various stages in the process being studied. Frank cites his group's work on the ribosome, which assembles from two separate subunits into an immature intermediate state and then into the mature structure. "That already gives us four different populations that are all intermixed always at any given time," he says. For that reason, he and others often analyse all time-point data together in a single batch rather than tackling each separately; they then use algorithmic analysis to work out how the distributions of protein conformations change over time and then reconstruct the underlying dynamic process.

Heterogeneity is an inherent problem in any cryo-EM experiment, notes Ellen Zhong, a computational biologist at Princeton University in New Jersey. "You have all these 2D projections, but the interesting thing is that each of the projections is a different molecule because the molecules are intrinsically dynamic," she says. The difference with time-resolved methods is that the goal is now to reconcile the selected 2D images into multiple intermediate states — and put those into the right sequence — rather than finding a single 'correct' answer, as with conventional cryo-EM.

Widely used software tools for conventional cryo-EM, such as RELION (which is open source) and CryoSPARC (free for academic users), are generally up to this task. But fitting these various structures into a biologically sound sequence of events remains difficult. In his team's actomyosin study, Muench found it essential to pair his structural-biology expertise with the deep knowledge of muscle biology provided by his collaborators. This allowed the team to ground its structural analyses in the context of mutations with known effects on muscle function or disease. "Without all that additional information, it's hard to interpret," says Muench.

Newer computational tools can fill in the gaps by fitting structures into a larger landscape of possible conformations. This can help to order the sequence of conformational states that a protein or complex transitions through during a given biological process. One such algorithm is CryoDRGN, developed by Zhong and her colleagues in 2021 when Zhong was a graduate student at the Massachusetts Institute of Technology in Cambridge[8]. She initially tried tackling this heterogeneity problem using methods such as molecular dynamics simulations, but that's "purely *in silico*", she says: "It's just a simulation, and oftentimes very far removed from reality. I wondered, 'can we use deep-learning techniques to learn complex continuous distributions?'" In one 2023 demonstration, Zhong and her collaborators used CryoDRGN to reconstruct the complex enzymatic process that underlies polyubiquitination, a protein modification that marks target molecules for destruction by the proteasome[9].

"We basically harvest most of the supercomputing power at Peking University."

Secrets of molecular choreography

Several dozen TR cryo-EM studies have been published to date — but they cover only a tiny fraction of the thousands of cryo-EM structures deposited in the Protein Data Bank. But researchers are already imagining the possibilities as the method gains momentum.

Areas such as drug discovery could be transformed by TR cryo-EM — for instance, by enabling the rational design of agents that account for molecular movement and that target or trap proteins in specific conformations. Enchev is particularly enthusiastic about opportunities to develop proteolysis-targeting chimaeras (PROTACs) — drugs that selectively modify and promote the degradation of disease-causing proteins. By revealing the often-fleeting interactions between a PROTAC and its target, TR cryo-EM could help to evolve ineffective or moderately successful drug candidates into potent medicines. "The real disruption would come if you could visualize these steps when they're failing and reason what makes them fail and how to change that," says Enchev.

Given enough examples, it should even be possible to extract fundamental principles of protein movement, which could guide the computational design of synthetic proteins that mirror the sophistication of real-world biomolecules. "If you can predict the motions, then you can maybe predict function," says Lorenz. "And if you can predict function, maybe you can design an artificial enzyme that catalyses a reaction that nature doesn't want to catalyse."

This is still well over the horizon, however. For one thing, the field lacks clear benchmarking standards. "We have time points, but we don't have error bars on our time points," says Enchev. It would be helpful if the field could identify well-defined experimental model proteins that can offer a benchmark for evaluating experimental and analytical methods, Enchev continues, and says he plans to make this a main focus at a TR cryo-EM meeting he will be hosting this month in London. Zhong and her colleagues are also interested in this problem, and have developed a freely available toolbox called CryoBench. It includes a suite of data sets, metrics and performance benchmarks for evaluating computational methods for reconstructing conformational ensembles from heterogeneous cryo-EM data.

But most importantly, the field needs more labs with the capacity to perform time-resolved experiments in-house. Method developers are eager to broaden the adoption of their systems, but manufacturers have been hesitant to license and commercialize technologies with such a small user base. Still, TR cryo-EM pioneers are hopeful. "This definitely needs a global collaboration instead of just a few labs studying a few systems," says Mao. "So many systems in the cell need such an approach, and we're not going to get it done by ourselves."

Michael Eisenstein is a science writer in Philadelphia, Pennsylvania.

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