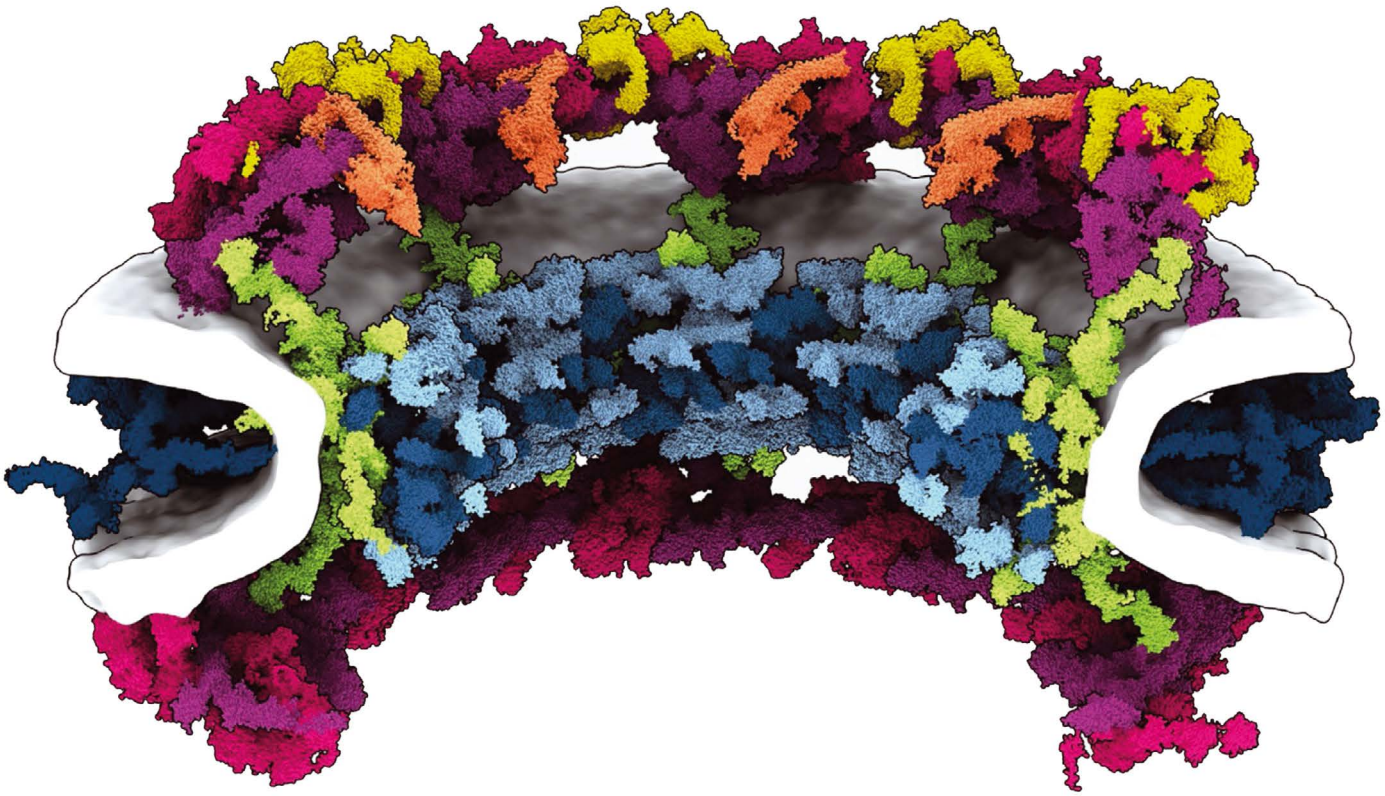


S. MOSALAGANTI *ET AL./SCIENCE*

Cryo-electron microscopy is revealing the inner workings of the nuclear pore complex.

A FLASH-FROZEN PEEK INSIDE THE CELL

Cryo-electron tomography is a hugely promising tool in visual proteomics – if researchers can work out what they are seeing. **By Michael Eisenstein**

Somebody hands you a tiny piece of a complex engine and asks you to explain its role in the larger machine. Unless you're a skilled mechanic or engineering savant, the best you can do is give an educated guess.

This, fundamentally, is the cell biologist's plight. Methods such as cryo-electron microscopy (cryo-EM) and the artificial intelligence system AlphaFold offer tools for deriving the atomic-scale structure of individual proteins; state-of-the-art molecular methods can illuminate where in the cell those proteins are active and what they do. But visualizing the real-world interplay of proteins in their native environment – the molecular-scale workings of the cellular engine – remains difficult. An emerging tool called cryo-electron tomography (cryo-ET) is helping to bridge that gap, however, and providing unprecedented opportunities to peek under the cellular hood.

In 2022, for instance, structural biologist Peijun Zhang at the University of Oxford, UK, and her colleagues used cryo-ET to unravel the enzymatic processes that certain bacteria use to capture and repurpose the greenhouse gas methane[1]. The researchers' analysis showed how two key enzymes collaborate by assembling into membrane-bound structures that directly funnel one enzyme's output to form the other's input. "The reaction product is all concentrated in that small compartment so that it would be effective," explains Zhang. This finding, she adds, could only have been made in the context of intact cells, in which the complexes are in their native forms. "You wouldn't be able to do any such stuff *in vitro*."

Other researchers are applying cryo-ET to gain insights into processes such as photosynthesis and protein production, and the workings of the nuclear pore complex (NPC) – a massive protein assembly that regulates molecular traffic in and out of the nucleus. This is all part of the emerging field of visual proteomics, which aims to describe the biomolecular infrastructure of cells with a resolution approaching that of *in vitro* methods such as cryo-EM. But cryo-ET is nowhere near that stage yet, and data from experiments that use it often defy easy interpretation.

"We see everything, so that comes with the opportunity to observe unexpected things," says Wolfgang Baumeister, who is a structural biologist at the Max Planck Institute of Biochemistry in Martinsried, Germany. "But it comes also with the huge challenge of identifying and annotating all the densities we see in the tomogram." And for now, researchers are still working out how best to discern the targets that interest them the most in the densely packed environment of the cell.

Dating to the 1930s, electron microscopy remains a powerful tool for visualizing biological samples at atomic resolution. In the variant known as cryo-EM, purified proteins are flash frozen into glass-clear ice and then imaged. The detailed pictures of these protein molecules, captured at different angles and orientations, are computationally reconstructed to yield 3D structures – sometimes with sufficient resolution to distinguish individual atoms.

Stacking the odds

Cryo-ET involves freezing whole cells rather than isolated proteins, and then using specialized equipment to 'mill' the top and bottom of the sample to produce a thin window known as a lamella. This is typically accomplished by rapidly scanning a focused ion beam over a target site to shave away excess ice and biological material. These lamellae are then tilted and imaged at multiple angles to document the molecular contents before undergoing algorithmic reconstruction into a 3D tomogram, followed by further processing and analysis.

The technique's roots can be traced back to Baumeister, whose lab began developing cryo-ET nearly 40 years ago. Getting the technique to deliver on its promise took decades of work, he says. "It was only less than 20 years ago that we could really do what we were hoping to do, which is look at the molecular architecture of cells." Those years have seen considerable methodological improvements, including the introduction of focused-ion-beam milling, ultrasensitive electron detectors and higher-quality microscopes.

Yet none of that makes cryo-ET easy. "It's not quite structural biology, and it's not quite cell biology – it's sort of like some hybrid in between," says Elizabeth Villa, a biophysicist at the University of California, San Diego. Unlike the purified protein samples used for cryo-EM, tomography entails searching for biological structures in their natural environment – with an emphasis on the 'search'. "The dirty secret of cryo-ET is that a tomogram covers like 0.001% of a mammalian cell," says Villa. The odds are therefore stacked against any individual lamella containing the specific protein or event that a researcher is seeking. The chances are better with microbial specimens – Villa estimates that researchers can cover roughly 30–50% of a bacterial cell with a tomogram – and microbiology is a major focus for cryo-ET researchers.

Still, specialists such as Villa recommend researchers do their homework before starting out – using other methods to understand how abundant a protein is, or the factors that influence the frequency with which an event occurs, for instance. This is especially important because cryo-ET requires hundreds or thousands of examples of the target of interest to produce an informative reconstruction.

Even then, studies can easily become fishing expeditions. In 2022, cell biologist Benjamin Engel at the University of Basel, Switzerland, completed a years-long effort to reconstruct the cellular machinery that the algae *Chlamydomonas* uses to assemble and maintain its cilia – hair-like structures involved in movement and environmental sensing[2]. "We were just milling these algae cells randomly, trying to hit this structure at the base of the cilium," says Engel. "It took many years to gather this one structure."

"We see everything, so that comes with the opportunity to observe unexpected things."

Faster lamella-generation workflows are making this effort less painful, and Zhang says that automation has increased the number of samples that can be prepared from 5 or 6 per day to 40. Still, solving a structure can require hundreds of samples.

Shining a cellular spotlight

Researchers can also narrow down the cellular terrain that they need to comb using a technique known as correlative light and electron microscopy (CLEM). With this, samples are fluorescently labelled to mark specific proteins or compartments in the cell in which an event of interest is most likely to occur prior to freezing. The sample is then imaged under a specially designed cryo-fluorescent microscope so that researchers can better target lamellae.

In 2020, for instance, Villa and her colleagues used CLEM to reveal how a mutated protein associated with Parkinson's disease interferes with the trafficking of essential biomolecules between different parts of the cell[3]. "Only 30% of the cells even had the phenotype that we were looking for, and if we had just done random lamellae in random places, we would've never found it," says Villa.

But as with all things cryo-ET, aligning fluorescence and electron-microscopy data isn't straightforward. The resolution produced by standard fluorescence imaging is so much lower than in electron microscopy that producing optimal lamellae can still require a lucky break. "Sometimes your whole image at electron-microscopy magnification would be just one pixel in the light microscope," explains structural biologist Kay Grünewald at the University of Oxford. Groups are working to merge CLEM with single-molecule-resolution fluorescence microscopy, but that remains a work in progress. For now, Grünewald and others often use tiny fluorescent beads that are electron-dense enough to be detected with an electron microscope, making it easier to triangulate locations between the two imaging systems.

But moving samples between multiple instruments creates its own difficulties, including increased opportunity for contamination. And Julia Mahamid, a structural biologist at the European Molecular Biology Laboratory in Heidelberg, Germany, notes that even a frozen sample can warp and shift during the preparation process. Without fluorescent imaging at this stage, these perturbations might go unnoticed.

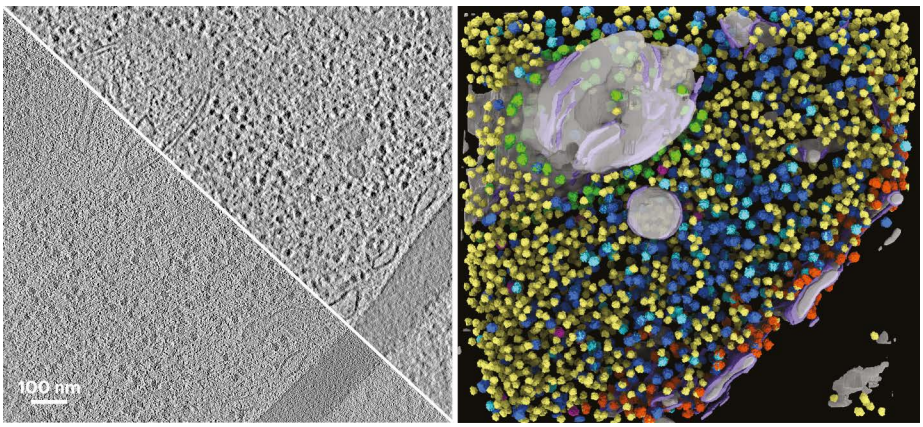
A new generation of integrated cryo-CLEM systems promises to ease these challenges. The ELI-TriScope platform[4], for instance, developed at the Chinese Academy of Sciences, Beijing, provides researchers with colocalized electron (E), light (L) and ion (I) beams. Structural biologist Fei Sun, one of the lead researchers on this effort, says that the system has taken a lot of the guesswork out of his study of the centrioles that sort and separate chromosomes during cell division. "Our success rate increased from previously around 5% to 90%," he says. "It's a huge improvement."

Meanwhile, because electron microscopy is missing an equivalent of green fluorescent protein – in which a genetically encoded reporter can be used for cellular labelling in fluorescence-imaging experiments – the hunt is under way for labelling methods that would allow researchers to home in on targets of interest without fluorescence imaging. Existing options, such as gold nanoparticles coupled to protein-binding functional groups, are too bulky and have the potential to artificially aggregate many targets at once. Baumeister is concerned that such tags could perturb the nanometre-scale details that cryo-ET is used to uncover. Nevertheless, some have seen promising results. For example, Grünewald and his colleagues have developed DNA-based 'origami' tags that fold into electron-dense asymmetrical shapes that bind and point towards cellular features of interest[5]. Although the tags are currently restricted to external proteins, Grünewald is exploring ways to deliver them into the cell itself. "It's not as versatile yet as we hope, but for certain questions it works quite well," he says.

Combing through the chaos

After the milling, tilting and imaging are done, cryo-ET images are reconstructed into a single 3D tomogram, and the real hard work begins. Scientists must pore over each tomogram to pluck out their cellular features of interest.

Unfortunately, even high-quality raw cryo-ET data resemble the grainy monochrome static of an untuned analogue television. Seasoned veterans can discern features such as lipid membranes or mitochondria, but finding individual proteins or complexes is a trickier proposition.



I. DE TERESA-TRUEBA *ET AL.*/*NATURE METHODS*

After data collection, the challenge with cryo-ET is analysis. Here, a 2D slice of a yeast cell (left panel; raw data at lower left, improved contrast at upper right) is transformed into ribosomal reconstructions (right panel) by the analysis tool DeePiCt.

The standard approach is to use template-matching software, which exploits existing structural data to pick out the target amid the whorls of black and grey. Although relatively fast, this approach can be sloppy, and Mahamid points out that even well-studied molecular assemblies such as the protein-synthesizing ribosome can slip through the net. The process is even less reliable for small, less abundant or disordered proteins – and, by definition, requires prior structural knowledge about the target.

More automation

Structure-prediction algorithms based on deep learning, such as AlphaFold and RoseTTAFold, have become valuable assets. They allow researchers to formulate hypotheses about what poorly understood proteins look like or even to reverse-engineer mysterious structures, and potentially identify the protein sequences that formed them. In June 2022, for instance, Martin Beck at the Max Planck Institute of Biophysics in Frankfurt, Germany, and his colleagues used AlphaFold and RoseTTAFold in a comprehensive investigation of the human NPC[6]. The resulting model captured multiple conformations that accounted for more than 90% of the around 1,000 proteins that form the NPC, many of which were poorly defined beforehand.

Deep learning is also helping researchers to annotate their tomograms in a more automated fashion. But these tools, too, often use templates, including the DeePiCt algorithm[7], developed by Mahamid and her colleagues. Mahamid describes it as a vast improvement over earlier template matching, but adds that "we're still missing about 20% of our particles".

In April, Stefan Raunser's team at the Max Planck Institute of Molecular Physiology in Dortmund, Germany, described an alternative approach called TomoTwin that can pick and classify particles without prior knowledge of the structure of interest[8]. According to Engel, this method goes a long way towards the goal of automatically generating detailed protein atlases of a sample. "The one thing I really think we all want is the 'visual proteomics' button: you press one button and it just gives you everything," he says.

Armed with enough high-quality particles, cryo-ET can generate structures that approach the atomic detail of cryo-EM. This typically involves a process called sub-tomogram averaging, which uses many 3D particle snapshots to produce a high-confidence consensus of what the target looks like. The final resolution of these reconstructions can be further enhanced by applying image-processing and refinement algorithms, such as the Warp and M tools developed by computational biologist Dimitry Tegunov at biotechnology firm Genentech in South San Francisco, California. These tools correct for experimental anomalies that reduce cryo-ET image quality.

> **"The one thing I really think we all want is the 'visual proteomics' button."**

In collaboration with researchers including Tegunov, Mahamid used these and other tools to reconstruct the interaction of a bacterial ribosome with an inhibitor of protein synthesis at 3.5-ångström resolution[9] – roughly three times the diameter of a hydrogen atom. This was no mean feat; Mahamid needed 20,000 ribosome examples to build this model, and ribosomes are among the more abundant complexes in the cell.

That, says Baumeister, is a crucial limitation. "Probably no more than 10–20% of the proteome is amenable to sub-tomogram averaging," he says. "It requires abundance, and it requires size." More automation and faster sample processing could address the former, he says, but smaller and more disordered proteins will probably remain a challenge until the quality of the raw image data improves considerably.

Towards visual proteomics

As cryo-ET specialists push the technical envelope, researchers worry that the field will devolve into a race for atomic resolution. "For a lot of the questions that we are addressing, we don't need it," explains Engel, although he adds, "I wouldn't turn it down." For example, much of Engel's research focuses on the internal machinery that drives photosynthesis in plants – specifically where these structures are located and how they are organized. Even a relatively modest resolution of 10–15 Å might be sufficient to address such questions, he says.

For now, what the cryo-ET field most urgently needs is data. Artificial intelligence algorithms are only as good as their training sets, and progress in deep learning for image processing and analysis will require a vast repository of annotated images that can guide the picking and interpretation of individual particles from the complex cellular soup.

More trained personnel with access to the highly specialized – and costly – equipment are also needed. Baumeister estimates that developing a full workflow for cryo-ET can cost upwards of €10 million (US$10.7 million). "That's much more than the start-up package of a typical young faculty member," he says. Indeed, when Mahamid – a former member of the Baumeister lab – first went looking for faculty jobs, her choices were limited by the need for existing cryo-ET capacity. "I could only apply to one place," she says.

Nevertheless, momentum is building, and researchers are finding creative ways to apply the method. This includes Engel who, with his collaborators, is using portable flash-freezing apparatus to collect marine specimens from European coastal sites for cryo-ET analysis at their lab in Basel. Such technology, coupled with next-generation computational tools, Engel says, could one day make it possible to go from raw sample to molecular inventories even for entirely new species. "If we can push the resolution improvements and if we can identify all the things through a combination of bioinformatics and imaging, we can really do anything," says Engel. "It opens up the door for so much more exploration of our world."

Michael Eisenstein is a science writer in Philadelphia, Pennsylvania.

1. Zhu, Y. *et al. Nature Commun.* **13**, 5221 (2022).
2. Van den Hoek, H. *et al. Science* **377**, 543–548 (2022).
3. Deniston, C. K. *et al. Nature* **588**, 344–349 (2020).
4. Li, S. *et al. Nature Methods* **20**, 276–283 (2023).
5. Silvester, E. *et al. Cell* **184**, 1110–1121 (2021).
6. Mosalaganti, S. *et al. Science* **376**, eabm9506 (2022).
7. de Teresa-Trueba, I. *et al. Nature Methods* **20**, 284–294 (2023).
8. Rice, G. *et al. Nature Methods* **20**, 871–880 (2023).
9. Tegunov, D. *et al. Nature Methods* **18**, 186–193 (2021).