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Calling cell biologists to try cryo-ET

Cryo-electron tomography (cryo-ET) advances can ease the path to 3D renderings of cellular architecture.

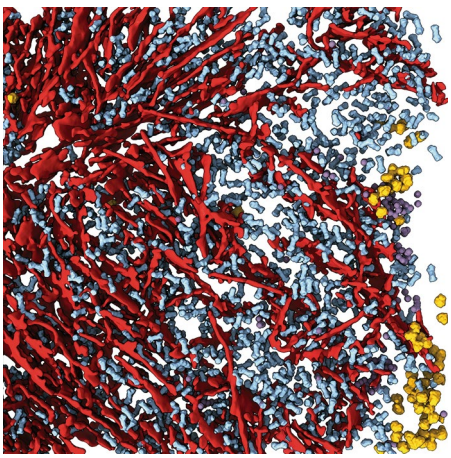
Vivien Marx

Plunge-freezing sounds like a summertime jump into a refreshingly cold lake. In the lab, plunge-freezing is a way to vitrify cells in a physiological, hydrated state and avoid staining or chemical fixation. High-pressure freezing is used with tissue or multicellular organisms. In all cases, the process is "the best possible structural preservation that can physically be achieved," says Wolfgang Baumeister, a researcher at the Max Planck Institute of Biochemistry who began working in cryo-electron tomography, or cryo-ET, in the late 1980s. Cryo-ET belongs to the Nobel-Prize-winning family of cryo-electron microscopy (cryo-EM) techniques[1,2].

When done well, speedy cooling outpaces the formation of ice crystals in the cell's watery interior. That ice would damage structures and disrupt subsequent imaging in an electron microscope, which is the next step in cryo-ET, says Elizabeth Villa, a researcher at the University of California, San Diego. When she shows the three-dimensional images generated with cryo-ET called tomograms to cell biologists, "they're blown away," she says. But there are barriers to entry: cryo-ET requires expertise and resources for acquiring, using and maintaining multi-million-dollar equipment. Eager to take down the barriers, the cryo-ET community is setting up small and big ways to help their cell biologists.

For instance, Villa is hiring staff who will tend to external scientists coming to her lab to collect cryo-ET data on their samples. Grant Jensen, a researcher at California Institute of Technology (Caltech), produced a YouTube video series on cryo-EM that is "probably one of the most impactful things I have ever done," he says.

With new Common Fund grants from the National Institutes of Health, he and, separately, researchers at Yale University, the University of Utah and Purdue University are building additional educational resources for newcomers; new cryo-EM access and training centers are being set up by The New York Structural Biology Center, Stanford University and Oregon Health and Science University among others. Biophysicist Sriram Subramaniam is leaving his NIH position to direct a national cryo-EM facility for Frederick National Laboratory for Cancer Research that is funded by the National Cancer Institute and run by the company Leidos. The national electron bioimaging center at the UK's Diamond Light Source lets researchers book sessions to collect cryo-ET data from their samples. At Diamond, scientists can also take part in workshops on cryo-ET sample prep, and microscope and software use. Such facilities provide labs with infrastructure and "there will be others, I think," says Baumeister. For example, he says, Chinese universities are investing heavily in this space.

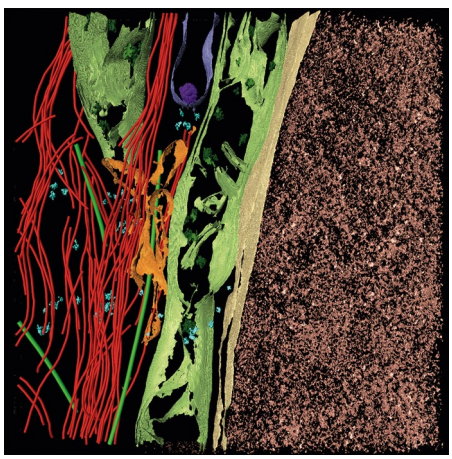


Cryo-ET can reveal 'molecular sociology.' This tomogram shows poly-Gly-Ala aggregates (red) and proteasomes (blue) where proteins are degraded. Protein aggregation is a hallmark of neurodegenerative disease. (Credit: Q. Guo, Baumeister Lab, MPI Martinsried.)

Akin to computed tomography in medicine, cryo-ET delivers computed 3D tomograms of cellular landscapes and structures at a resolution of around four nanometers. These tomograms are based on many images that capture a sample's deflection of an electron beam. The images are aligned with software tools, for example, IMOD. By applying post-processing steps such as subtomogram averaging and a few tricks of the trade, the technique can reach sub-nanometer resolution, says Baumeister. Cryo-ET can also be correlated with fluorescence-microscopy-based live-cell imaging.

Trained as a physicist, Villa's love of cryo-ET arose during a physiology course at the Marine Biological Laboratory and she vowed to never look at isolated molecules again. As a postdoctoral fellow in Baumeister's lab she learned more about how cryo-ET can, in his words, capture the "molecular sociology" of cells, show cellular structures and molecules in the context of interactions and influences that shape function. As she studies when and how proteins talk to their 'friends', Villa says she feels like a "molecular anthropologist." "Proteins are very 'social animals,'" she says. At any given time, proteins can belong to a number of different complexes.

Cryo-ET is not easy, quick or cheap, and Villa reminds her students that 'cry' is part of 'cryo.' Experiments often fail, "we fail more," she says. She and other cryo-ET methods developers want to show that the method is more than a challenging path to a pretty picture. Cryo-ET brings to cell biology and structural biology a "wild concept," says Subramaniam: a way to peer inside cells and see proteins in situ at high resolution and in 3D. Instead of looking at proteins one by one, isolated from the cell, purified and crystallized, cryo-ET shows



A tomogram of a human U2OS cell milled into a thin lamella for imaging. Actin filaments (red), lysosome (purple), microtubules (green), nucleoskeleton (pink), mitochondria (light green), nuclear envelope (beige), ribosomes (cyan), rough endoplasmic reticulum (orange). (Credit: R. Watanabe, Villa lab at UCSD)

proteins "when they're doing their normal job," he says. "The potential to look at things in the cell as they are, that's the promise, that's what excites cell biologists at large."

Daggers and other sights

When a lab can purify tens of thousands of identical copies of a structure such as ribosome, a high-resolution approach such as single-particle reconstruction using cryo-EM might work best. If the object under study is unique, perhaps owing to activities being captured there, in Jensen's view cryo-ET is the method of choice.

Electrons can destroy samples but cryo-ET involves electrons shot at low dosage. Imaging of vitrified samples with transmission electron microscopy (TEM) is performed as a tilt series: each image is taken at a slightly different angle, and the stack is then computationally merged into a tomogram. Across the cryo-EM family, "the mathematics of how you merge the images are deeply related," says Jensen. Learning cryo-ET well takes around a year in his lab, where many have an x-ray crystallography background. Along with electron microscopy know-how, it takes dexterity to handle grids on which cells are grown, then plunge-frozen and imaged.

Not all cell types grow readily on this grid. "You want them to grow really flat," says Jensen. Plunge-freeze too quickly and the grid gets bent; plunge too slowly and ice crystals form. A sample's edge might be fine but ice crystals might have formed in the center where freezing progressed more slowly, says Villa.

Much cryo-ET work has involved bacterial and archaeal cells, which deliver thin samples. Using cryo-ET, Grant Jensen and his team obtained the complete structure of *Treponema primitia*'s flagellar motor, a nanomachine with 25 different proteins. The tomogram at seven-nanometer resolution indicated how the stator, the membrane-embedded torque-generating section of the motor, works with the spinning rotor. It was hard to get this insight with other techniques, because the stator tends to not purify with the rotor.

Jensen's favorite cryo-ET-based discovery came with *Vibrio cholerae* cells, the cholera-causing species of *Vibrio*[3,4]. The lab set out to study chromosome segregation in these bacteria. "Instead of seeing that, we saw these beautiful elaborate tubes," he says. The sheaths—around 12 and 15 nanometers across and 300 nanometers long—were unknown. In showing the images around, he contacted Harvard Medical School researcher John Mekalanos, who studies bacterial virulence with biochemical and genetic methods, also in several *Vibrio* species. "We wondered if maybe the tubes we were seeing were actually the machine that allowed them to kill," says Jensen.

The researchers decided on CLEM, correlated light and electron microscopy. They tagged a protein in the bacteria's VI secretion system with green fluorescent protein, imaged with fluorescence light microscopy followed by cryo-ET. The sheaths turned out to be spring-loaded, "poison-tipped molecular daggers," says Jensen. *Vibrio* uses these daggers to send toxic proteins into a prey cell, such as another bacterium or a eukaryotic cell. Cryo-ET had imaged parts of the dagger in two states: assembled and disassembled.

"His pictures and our pictures just completely revealed basically what this machine was and how it worked," says Jensen. The tomogram showed how the pieces fit together into a whole and labs are now studying this machine at higher resolution such as with single particle reconstruction.

In collaboration with colleagues at UCSD and University of California San Francisco, Villa and her team went back and forth between fluorescence microscopy and cryo-ET to study a bacteriophage right as it infected a bacterium. Given how hard it is to image bacterial substructures in light microscopy, the tomograms offered guidance. The researchers saw a nucleus-like structure that neither bacteria nor phages have[5]. It turns out to be where phages are delivered and then loaded with DNA. "It's like a little factory of viruses," says Villa. The 'factory' might shield phages from the bacterial immune system.

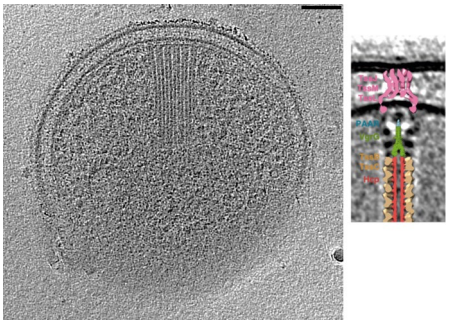
Moving from fluorescence microscopy to electron microscopy is like seeing a distant spot in a night-time cityscape and trying to find that very spot during the day. (Credit: Left: Dong Wenjie/Moment/Getty; right, AbZoLuteWin/iStock/Getty Images Plus)

Cryo-ET brings to cell biology a way to peer inside cells, see proteins in situ at high resolution and in 3D, says Sriram Subramaniam. (NIH)

Getting eukaryotic

Transferring coordinates from light microscopy to EM means traversing huge scales. A typical cell's area covers several hundred square micrometers; a tomogram's field of view is around one square micrometer, says Baumeister. Villa is working on a bridge-scaling workflow for moving from live-cell imaging to cryo-ET. When an event of interest occurs, perhaps when stimulating cells with optogenetics or studying migrating cells, a researcher will be able to say "freeze" and then zoom in with EM. Instead of the typical cryo-EM grid, her lab prefers to grow cells on silicon nitride. The material can be patterned, for example, with grooves for cell-migration experiments. She uses scanning electron microscopy (SEM), which images surfaces, as well as TEM, which images through samples.

Subramaniam and his team developed computational ways to link live-cell imaging and SEM of the same region. An SEM stack for generating 3D images is massive, says Subramaniam. In the SEM images for an HIV-focused project, there were many virus-like objects. "99.9% are false positives



Vibrio cholerae deploys a poison-loaded dagger. It was discovered using fluorescence microscopy and cryo-ET. (Credit: Jensen lab, Caltech; Mekalanos lab, Harvard Medical School; right: adapted with permission from ref. [4])

because lots of things look like little blobs in the cell," he says. Applying CLEM, they used landmarks and two-color imaging to locate the viruses in their images. They developed and used a segmentation algorithm that became part of ITK, an open-source image analysis toolkit.

Scale-toggling remains challenging: it's like being interested in one bright spot in a night-time satellite image of a city and then being dropped somewhere in that city during the day and needing to find that spot, says Villa. She and others are exploring approaches that could help: ways to label cells so they "light up" in EM; they might try doped GFP, self-arranging, designed tags, or other ways of tagging proteins.

When doing cryo-ET with eukaryotic cells, the Jensen lab has encountered auto-fluorescence. Structures with multiple membranes, such as multi-lamellar bodies and secretory granules, autofluoresce at 80 Kelvin more than at room temperature, says Jensen. His team developed a way to deconvolve fluorescent tags from this autofluorescence background: they record images from labeled and unlabeled cells in two fluorescent channels, one of which corresponds to the tag color and the other is from an unrelated channel.

Typical fluorescence light microscopy at room temperature involves lenses with high numerical aperture and oil-immersion lenses. "We can't do that when our sample is frozen," says Jensen. Instead, the team must use air objective lenses with long working distances that provide lower resolution. This led them to develop and use cryo-PALM, combining photoactivated localization microscopy and cryo-ET[6].

Using human induced pluripotent stem cells, Villa is collaborating with UCSD colleague Susan Taylor and the Studer lab at Memorial Sloan Kettering Cancer Center; they are applying cryo-ET and other techniques to study molecular aspects in Parkinson's disease. The team is looking at cellular changes caused by LRRK2, a protein with a role in a rare heritable version of Parkinson's disease. Perhaps, says Villa, microtubule-based transport blockage plays a role in the disease.

CLEM with cryo-EM, including cryo-ET, will be a boon to cell biology, says Jensen. Eventually, it will be routine to identify a moment of interest and immediately move to EM to see the molecule, its conformation and all around it. Labs could revisit fluorescence experiments, work with bacteria, viruses, eukaryotic cells, "almost anything," he says. The technologies for doing so are rapidly developing. Some remaining challenges are how to best retrieve cells from tissues and ready them for cryo-ET and how to raise experimental throughput.

Currently, a tilt series takes tens of minutes. "We're now testing a stage which may allow us to get good tilt series in tens of seconds," says Jensen, emphasizing the word "may." This stage could let labs use cryo-ET in projects typically done with cryo-EM-based single-particle reconstruction. A tilt series would deliver more information than a single projection, he says, noting that the lines between different types of cryo-EM are blurring. Combining approaches will help with a number of issues such as disambiguating a structural change that looks like a conformational change but is just an image taken from a slightly different perspective.

Detectors

Jensen's idea of accelerated tilt series will give detectors plenty to do. Cameras will have to read out and save data continuously, says Benjamin Bammes, who leads application development at Direct Electron, which makes detectors. The approach could increase throughput in cryo-ET but needs faster data processing. To accelerate writing detector data to disk, his company works on improving hardware and on compression algorithms.

Once she learned about cryo-ET, Elizabeth Villa vowed to never look at isolated molecules again.

(UCSD)

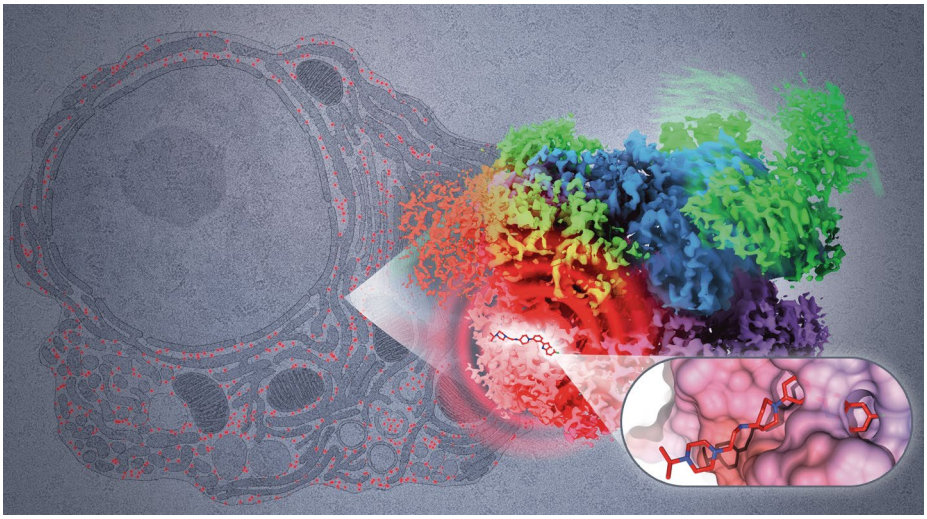
In cryo-ET, advances in detector technology matter for both SEM and TEM. Originally, detectors used photographic film, which provided a large field of view but no way to see images during experiments. Charge-coupled devices (CCDs) offered quick feedback but had issues with image quality and field of view, says Bammes. Direct detectors of electrons are faster and more sensitive and generate images directly from electrons, unlike CCDs, in which electrons are converted to photons. Gatan, now in the process of being acquired by Thermo Fisher Scientific, makes several direct detectors of electrons. EM instruments by FEI, which is owned by Thermo Fisher Scientific, have a detector called Falcon, which detects electrons directly. Detectors can usually be installed on microscopes from different manufacturers.

The field of view in detectors is steadily expanding. One model of Direct Electron's detector reaches 67 megapixels; Gatan's K3 has 24 megapixels. Bammes says his company is looking into expanding to 12,000 × 12,000k; company scientists are testing if the greater size risks edge distortion. A larger field of view is helpful for correlative approaches so labs can find the area of interest in EM detected in a fluorescence microscope.

Cryo-ET is a battle against noise. Every electron generates variable energy on the sensor, a variability that detectors normalize to improve image quality, says Bammes. Some electrons scatter off the sensor and light up several pixels, which creates irregularly shaped blobs instead of a "nice, discrete spot," he says. His company is exploring how larger pixels might help to reduce ambiguity in image analysis given that electrons would scatter only within that pixel. To handle irregularly scattering electrons that are more frequent in thicker samples, Villa uses an energy filter to harvest only the electrons she wants for imaging.

Thick and thinner

Baumeister's lab has long focused on proteasomes, protein complexes in eukaryotic and archaeal cells where proteins are degraded and recycled. He and his team have gone from studying them in isolation with X-ray crystallography and EM-based single-particle analysis to seeing them in a cellular context with cryo-ET. In recent work on proteasomes in rat cortical neurons, the team applied, among other techniques, flow cytometry, CLEM and cryo-FIB/SEM, which involves a focused ion beam machine for thinning samples[7]. With many samples, it's still a long road from vitrification to the microscope, says Baumeister. But given the workflows in



Within a decade, says Sriram Subramaniam, labs might be able to image a drug as it interacts with cellular proteins. (Credit: S. Subramaniam, V. Falconieri, NIH)

development, that route will shorten and labs will have an easier time with a wide diversity of samples, including eukaryotic cells. Thinning techniques have played a big role in cryo-ET, he says.

FIB milling is a technology that comes to the life sciences by way of the semiconductor industry, says Subramaniam. Coordinates from fluorescence microscopy are transferred to an FIB instrument that performs electron microscopy after milling samples, thinning them at a desired location. Especially for TEM, samples must be milled into thin lamellae for imaging. Baumeister, Subramaniam and other researchers have helped bring FIB to the life sciences[8,9]. Subramaniam has collaborated with microscope manufacturers FEI and Zeiss to advance the technology that led to commercial instruments.

Currently a tilt series takes tens of minutes. But there may be a way to cut that to tens of seconds, says Grant Jensen.

(Caltech)

Originally, labs applied milling to lift a section of interest out of a sample, says Subramaniam, which is a like cutting a section out of the yolk in a fried egg. Since then, labs, including his at NIH, have milled samples by cutting away the sides, milling the area of interest while keeping that region attached to the sample. With FIB, gallium ions are sputtered across a sample surface, shaving off a layer at a time. After each shave, the surface is imaged. FIB made it possible to apply SEM for collecting an image series of a large area, he says. One issue, however, is that the imaging must be refocused as the shaving progresses. He and his team developed computational ways to keep the sample in focus throughout the milling process.

FIB has become highly refined. "Now we can take five nanometers off at a time, with the ion beam," says Subramaniam. He and his team have also developed algorithms to reduce image noise. Using FIB-SEM, he and his team generated details of HIV infection at 20-ångström resolution. The scientists imaged the synaptic mesh between HIV and T cells and the 'spikes' the virus uses to infect other cells. To get a big picture and to "walk through" cells, SEM is a quick approach, he says. With TEM, much higher resolution is possible at the moment, mainly because the microscopes differ, he says. Labs might start with SEM and follow up with cryo-ET using TEM, for example, to further analyze the tomograms and understand the role of a substructure in a cell.

Just look at it

Like many in this field, Subramaniam sees a bright future for cryo-ET and believes it and other cryo-EM techniques deserve to be more accessible. He wants to help grow the community, also through activities at the new facility he will lead. In five to ten years, methods might allow labs to visualize a drug latching onto proteins inside a cell. Baumeister wishes the cryo-ET field had exploded sooner but is gratified that it has finally come to be.

Electrons are better than X-rays for delivering structural information and with less damage, says Jensen. Although some technical challenges still need addressing, he believes that the future of structural biology is "all cryo-tomography all the time." That might be a slight exaggeration, says Baumeister, but he agrees with the sentiment. "This is why we pushed it and developed it," he says of the technology. "I really believe, yes, the future of structural biology is to study things in the context of the cell, preserving functional interactions."

Baumeister's vision is of "structural biology in situ" and routinely achieves three- to four-ångström resolution. "It's near-atomic resolution," he says, one at which labs can see alpha helices that will let them interpret many cellular events. Jensen likes to quote physicist and Nobel laureate Richard Feynman, who stated in a talk to the American Physical Society: "It is very easy to answer many of these biological questions, you just look at the thing." ❐

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