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Zooming in on electron tomography

Electron microscopy has emerged as an important tool for determining the three-dimensional (3D) structures of cellular components. Advances in instrumentation, methods for data collection and techniques for computation are improving the resolution and breadth of application for this powerful technology. Laura Bonetta reports.

From the 1950s to the 1970s the electron microscope (EM) offered a close-up view of the structures inside the cell, revealing unprecedented detail. "But once those issues were largely resolved, the EM moved to the sidelines," says Richard McIntosh, who directs the Laboratory for 3D Electron Microscopy of Cells at the University of Colorado at Boulder. "With good fluorescent optics and tags, the light microscope became a fantastically powerful tool." While temporarily out of the limelight, the electron microscopy field was not at rest. During this time substantial technical improvements were made in specimen preparation, instrumentation and software, allowing the EM to reemerge as an invaluable tool for analyzing molecular complexes.

In recent years the EM has been used with great success to reconstruct 3D forms of cellular components at a resolution that falls between that of atomic structures defined by X-ray crystallography and more global cellular patterns observed with the light microscope. The ability of the EM to fill this gap, along with the ease and speed with which newer instruments can be used, have given way to what many in the field are quietly referring to as a 'revolution'.

"The rapidly growing interest in 3D EM imaging is exemplified by a recent initiative of the European Commission to establish a network of excellence," says Abraham (Bram) J. Koster at Utrecht University. The 3D-EM Network of Excellence, funded by a grant of ten million euros, aims to foster collaboration among the leading European laboratories in electron microscopy to further develop the methodology and make 3D imaging infrastructure more accessible to nonspecialists (see http://www.3dem-noe.org/).

EM basics

The two most common types of EMs available commercially are the transmission electron microscope (TEM) and the scanning electron microscope (SEM). With the SEM a 3D image of the specimen's surface is produced, whereas the TEM reveals more information about internal structure. As a result, the TEM is the favored tool of the biologist.

TEM imaging involves several steps, some of which are completely automated. A sample, either frozen or embedded in plastic, sits on a metal grid that is clamped into a removable stage in the column of the microscope. A high-energy electron beam passes down this column at a speed that depends on the accelerating voltage of the microscope. Several electromagnetic lenses focus the electron beam and image the electrons that pass through the sample onto photographic film or a charge-coupled device (CCD) camera.

As the electron beam traverses the sample, features from different levels within the object become superimposed in the resulting two-dimensional (2D) image. Several techniques allow scientists to reconstruct 3D models from such images. One class of techniques can be applied to determining the structures of single, isolated molecules and the other is more amenable to larger and more complex biological structures.

Getting from 2D to 3D

Electron crystallography and helical reconstruction are two methods used to elucidate the structure of ordered arrangements of molecules, whereas single-particle reconstruction is used for individual molecules. The latter technique, pioneered by Joachim Frank, a Howard Hughes Medical Institute investigator at the Wadsworth Center in Albany, New York, and director of the Resource for the Visualization of Biological Complexity, entails freezing molecules in a thin layer of water on a grid and then taking hundreds of micrographs of the sample, each of which may contain hundreds of identical molecules in many different orientations. A software program then analyzes the images, picking out individual particles that are similarly oriented and averaging their images to improve the available

A Tecnai Polara transmission electron microscope. (Courtesy of FEI Company.)

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signal-to-noise ratio. Many such images, which represent views from different orientations, are then combined to reconstruct the molecule in 3D.

In a landmark study by Gabashvili *et al*. the ribosome was viewed at a resolution of 11.5 Å using 73,000 projections[1]. Since then, Frank's group has been able to push the resolution even higher to 7.8 Å.

Although it is extremely powerful, there is no question that the technique is labor intensive. "We solved the ribosome structure from 130,000 particles," says Frank. "If you consider that you get on average 500 good particles on a single micrograph, you can calculate how many images we had to take."

Gatan Model 924 cryotomography specimen holder. (Courtesy of Gatan, Inc.)

Investigators in several groups, including Bridget Carragher and Clint Potter of the Automated Molecular Imaging Group at The Scripps Research Institute in La Jolla, California, are working to 'automate the pipeline' so that more images can be taken in less time, thereby improving resolution. "We can acquire images of 100,000 particles in a day from a single sample, but it then takes many months to process the images," says Carragher.

However, most organelles and cellular substructures are too large and too variable for single-particle averaging approaches. For these applications, the technique of choice is electron tomography (ET). "The strength of ET is that it can deal with larger and more complex structures than X-ray crystallography and single-particle analysis," says Wolfgang Baumeister, director of the Max Planck Institute of Biochemistry in Martinsried.

Whereas single-particle reconstruction requires the analysis of a large number of identical structures because it relies heavily on averaging, ET can deal with unique structures. However, there is a price to pay for this capability. "Images obtained by tomography have poor signal-to-noise ratio, requiring, in turn, sophisticated image analysis tools for their interpretation," says Baumeister.

At the cutting edge

Tomography uses thicker samples (usually 100 nm to 1 μm than those used for conventional electron microscopy to provide more information in the depth dimension. The specimen sits on a special tilting stage and a series of digitized images is acquired as the specimen is tilted in 1- to 2- degree angular increments over ±70 degrees about a fixed axis. Following data collection the series of images must be aligned with respect to one another and the 3D structure computed.

A fundamental problem in ET is how to reconcile two conflicting requirements. The first is that to obtain a detailed reconstitution of an object, the scientist needs to obtain as many tilt images as possible, covering the widest possible angular range with the smallest possible increments. At the same time, however, the cumulative electron dose must remain within tolerable limits to prevent radiation damage to the sample. Until recently, this problem prevented the application of ET to frozen-hydrated samples (cryoET) which are quite sensitive to such damage.

The development of procedures for automated data collection (see 'Look, no hands' below) constitutes a major technical advance. These methods have reduced the necessary electron dose to the specimen to the point that cryoET has become practical. Frozen samples have the advantage that they are free of fixatives and stains and are thus believed to represent a near-native state. "CryoET is now at a point that it is broadly useful to cell biologists," says David A. Agard, Howard Hughes Medical Institute investigator at the University of California, San Francisco.

"We take three to five tomograms a day," says Koster. "The state of the art is to com-

plete the data collection in 20 minutes and have a 3D image one or two hours later. Of course you might need more images, and depending on the complexity of the problem, the analysis can still take a considerable amount of time."

A celebrated paper published in 2002 by Wolfgang Baumeister's group describes the use of ET of frozen *Dictyostelium discoideum* cells to study the actin cytoskeleton without first having to remove membranes or extract soluble proteins[2]. This allowed the scientists to analyze the cross-linking of individual actin filaments, their branching angles and membrane attachment sites. In addition, at a resolution of 5–6 nm, single macromolecules with distinct shapes, such as the 26S proteasome, were visualized in their cellular environment.

More recently, the group used the technique to hone in on the nuclear pore[3]. Their study identified the nuclear pore structure as having two main states, an important first step in beginning to understand how it works in a dynamic sense. "The dream experiment is to take snapshots of the nuclear pore complex at different times and map the trajectory of cargo going through it," says Baumeister. "We have to repeat the experiment with well-defined cargo that has been labeled so that we can follow it as it moves. Also we have to collect many more images."

A limitation of current cryoET methods is that the resolution is still not sufficiently high for visualizing many macromolecules and proteins. "The resolution of cryoET is generally about 5–10 nm, although Baumeister's group has been able to drive it to better than 4 nm," says Agard. "We could recognize macromolecules by tomography and map the entire proteome, but we need to get to 2 or 3 nm resolution for this dream to happen," says Baumeister.

Of cells and tissues

As technical development continues, cryoET promises to become the preferred method of choice for imaging isolated organelles and small cells. But samples larger than 500 nm to 1 μm cannot be visualized in their entirety by cryoET. To study such specimens scientists would have to take several serial slices of a sample to reconstruct the whole. The stumbling block is the inability to routinely cut good-quality frozen slices.

BOX 1 THE TEM TOOLBOX

The new generation of TEMs, provided by companies like FEI, JEOL and LEO (now owned by Zeiss), have higher accelerating voltage (that is, in the 200–400 kV range), a requirement for imaging thicker sections, and they are equipped with energy filters, another desirable feature for tomographic studies.

A thicker sample causes more electrons to scatter, affecting the quality of the images. The adverse contributions of these inelastically scattered electrons can be partially removed by energy filters. "Our filter is mounted below the table of a TEM, so it is often referred to as a 'post-column' filter. The biggest benefit for 3D cryo-TEM tomography is that the filter improves image contrast substantially to allow details in the sample, otherwise nonobservable, to be seen or captured by a CCD camera," says Ming Pan, marketing director at Gatan, Inc.

"In terms of technology, important requirements of the microscope are robustness and stability, both at the ambient and cryo temperatures, highly coherent electron sources, low dose capabilities and dedicated software allowing fully automated tomography and single particle analysis," says FEI's Edlands.

All modern microscopes come with software to control the microscope itself and the stage, including motorized tilt stages. The software also communicates to an image-recording device, such as a CCD camera. Because data must be in digital form for computation of the reconstruction, direct digital recording is a considerable time saver. It also obviates the need to interrupt data collection for film changes and processing. There are many manufacturers of CCD cameras to choose from, including Gatan, Inc. and TVIPS. Several companies manufacture specimen holders. The ones made specifically for tomography allow samples to be rotated in a single or dual axis, and some keep specimens at liquid nitrogen or even liquid helium temperatures.

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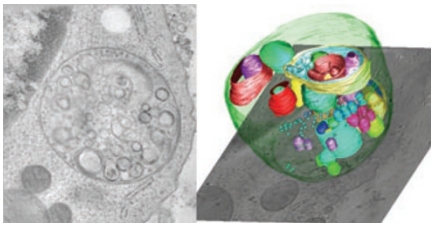


Images of a multilysosomal body. The left panel shows a conventional TEM image; the right, the complex 3D arrangement of the various membrane-bound structures in the interior of the organelle after electron tomography and modelling. (Courtesy of A.J. Koster and W.J.C. Geerts, Utrecht University, Utrecht.)

"The success rate with frozen sections is very low, probably 10% or lower," says Frank, who is working on refining the technique. "We are experimenting with different kinds of knives and using ion milling to move away from the knife altogether," he adds. Despite these difficulties, Frank says his group has been successful at obtaining tomograms of frozen, sectioned cells.

As these techniques are being developed, others are taking advantage of different approaches. "An alternative to cryoultramicrotomy of eukaryotic cell cultures and tissue specimens is rapid freezing followed by freeze-substitution with an organic solvent," says McIntosh. "Heavy metal salts and fixatives can then be added to the solvent." Rapid freezing helps to preserve the structure of cells and moving the sample into plastic makes the specimen hardier, allowing a scientist to cut big cells into samples suitable for ET. "We can cut many serial sections 300 nm thick. By collecting tilt series from such serial sections, we can collect multiple serial tomograms and assemble the result into reconstructions that include tens of cubic micrometers of cellular volume," says McIntosh.

The technology can be extremely powerful. "Electron microscopy is at the highest-resolution limit of a spectrum of complementary morphological techniques. When combined with molecular detection methods, ET is the only technique with sufficient 3D resolution to localize proteins to small membrane subdomains in the context of the cell," says Koster. In collaboration with different groups, including Judith Klumperman's and Koert Burger's groups in Holland and Alberto Luini's group in Italy, Koster used ET, combined with specialized labeling methods, to study secretory traffic in Golgi stacks[4]. "In a model system, we show that endoplasmic-reticulum-to-Golgi carriers join a Golgi stack by fusing with *cis* cisternae and induce the formation of intercisternal tubules, through which they redistribute their contents throughout the stack," says Koster. "These findings provide a new view of secretory traffic that includes dynamic intercompartment continuities as key players."

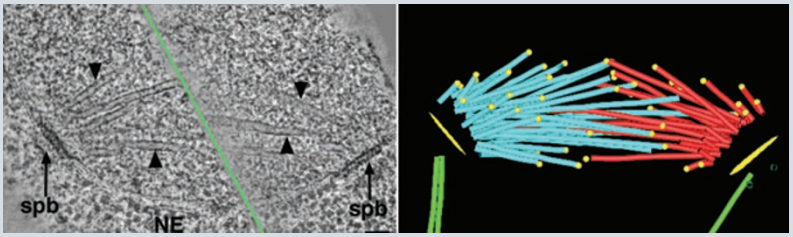
But the technique is not without its potential pitfalls. "The concern about adding fixative is that you are changing some structure at high resolution," says Koster. "This is always a debate."

Two is better than one

The path of the electron beam through the specimen increases as the specimen is tilted. As a result, it is usually not feasible to obtain good-quality images from sections at tilt angles above 60–70 degrees.

BOX 2 WE'LL DO IT FOR YOU

Although electron microscopy has become more user friendly, many researchers don't have access to an EM facility. As a result several companies, such as Paragon Bioservices, Advanced Biotechnology Inc. (ABI) and Charles River Laboratories Pathology Associates, provide electron microscopy as a service. "Our main clients are experimental labs doing preclinical work," says Jamie Whitman, director of the EM facility at ABI, which provides TEM services primarily for the identification of viruses and the quantification of virus in a sample. "We are pretty busy because there are few commercial labs that provide this kind of service." None of these companies have, however, approached the field of ET.



Serial tomography of yeast spindles. Left, single tomographic slice showing microtubules (arrowheads), spindle pole bodies (spb) and nuclear envelope (NE). Green line represents boundary between two serial tomographic volumes. Right, projected 3D model displaying spindle microtubules (blue, red) and their ends (yellow spheres), as well as the position of the spindle pole bodies (yellow) and cytoplasmic microtubules (green). (Courtesy of Richard McIntosh and Eileen O'Toole, University of Colorado at Boulder.)

Several NIH Biotechnological Resources were set up specifically for ET, including those at the University of Colorado at Boulder, at the University of California in San Diego, the Resource for the Visualization of Biological Complexity at the Wadsworth Center in Albany and the Keck Advanced Microscopy Laboratory at the University of California in San Francisco. "We do a limited amount of service," says Richard McIntosh, director of the Boulder facility. The lab also hosts visiting scientists who want to learn EM tomography and how to use the 3D modeling software package IMOD. "People come for four days and go home with several months worth of data to analyze," says McIntosh.

Sidec Technologies offers its expertise as a service to the pharmaceutical industry. "Now for the first time the pharmaceutical industry is interested in molecular tomography; they want to look at the 3D structure of proteins," says Ulf Skoglund, who developed the COMET algorithm used at Sidec. "Our experience is that you can have the complete 3D structure in one and one half months to complete all the paperwork for pharmaceutical industry," he adds.

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In practical terms, this means that most tomographic tilt series have a wedge of missing data corresponding to the angular range between the maximal tilt angle at which data were collected and 90 degrees. This 'missing wedge' causes a distortion in the 3D reconstruction.

The problem can be reduced by rotating the specimen by 90 degrees and collecting a second set of data at this orientation. This so-called dual-axis data collection scheme reduces the missing data from a wedge to a pyramid, but it also introduces new problems. "When a plastic specimen is first hit by electrons it collapses by as much as 40% along the beam axis. Additional radiation induces further change in the specimen, so two successive tomograms of the same sample are not identical. We have developed software to merge the two data sets together," says McIntosh. Another practical problem is the availability of instrumentation. "The options for obtaining dual-axis tilt series are limited," he adds.

McIntosh and his collaborators have used dual-axis ET to examine the 3D architecture of the Golgi system in rapidly frozen, freeze-substituted mouse pancreatic cells at a 6-nm resolution[5]. They were able to observe the presence of direct connections between Golgi cisternae that 'bypass' interceding cisternae when cells are stimulated with glucose to secrete insulin. These connections seem to provide a continuous passageway to facilitate the rapid transit of newly made proteins for secretion. "The excitement in this structure is that it shows a reorganization of membrane compartments when cells need to secrete insulin rapidly," says McIntosh.

Look, no hands

The advent of computer-controlled TEMs in the late 1980s and the availability of large-area CCD cameras (**Box 1**) has made automated image acquisition procedures possible. But the process is not as straightforward as it sounds.

Mechanical imperfections of the specimen stage and the inability to precisely set the eucentric height of the specimen relative to the tilt axis result in image displacements and focus changes. To correct these effects manually, the user has to recenter and refocus the specimen after each tilt increment, particularly at higher tilt angles, resulting in a substantial increase in the electron dose to the specimen.

In the past decade EM manufacturers have been able to take the user out of the picture by creating software that automatically corrects for these imperfections. For example, Tietz Video and Image Processing Systems (TVIPS) has developed a software solution that is compatible with nearly all TEMs that are equipped with remote- and computer-controlled stages. These procedures minimize the electron dose applied to the specimen as all corrections can be performed on areas adjacent to the region of interest for image acquisition.

At the same time, many academic labs are continuing to develop and perfect

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their own software for automating data acquisition. David Agard and John Sedat's groups at the University of California, San Francisco have taken an approach based on predicting how the image will move as tomographic images are obtained. "We adaptively learn about the process with each image," Agard says. "Microscope companies have focused on the idiosyncratic behavior of the stage and tried to fix that. We think the sample noneucentricity is the problem." Because Agard and Sedat's method depends on refining the predicted model as data are obtained, "no extra images are taken," he explains. His software, which is freely available, now works on FEI's Tecnai microscopes equipped with either Gatan or TVIPS cameras. "We are getting very close to a wall in terms of what we can do in terms of data acquisition," says Agard.

Reconstructing images

Because electron doses need to be kept low for ET, individual images are difficult to judge. To help, researchers have developed software to align series of images and reconstruct a picture. Automated alignment software often uses gold particles embedded in the sample to align one image with the next. Agard's group, on the other hand, has developed a software that relies on features within the image itself. "Gold beads can move around in a sample, even when it is frozen," says Agard. "We use a method that is very robust but quite computationally intensive."

Once images are aligned, dozens of software packages are available, most of them for free, to help reconstruct the 3D structure. More general packages, such as SPIDER, developed at the Wadsworth Center, include tools for 3D image reconstruction, as well as tools for 2D and 3D image processing. Others, including Boulder's IMOD are more specifically focused on reconstructing tomographic images and modeling. "We have a whole suite of tools, including a graphical user interface to make it easier to use," says McIntosh. IMOD is freely available and scientists can receive training on how to use it (**Box 2**).

Although, image alignment and crude 3D reconstruction steps are largely automated, human intervention is still needed to solve more complex problems. "When we look at larger volumes by serial sections and then do tomography on each section, one of the problems we encounter is how to recognize features of biological interest in the final image and follow them throughout. We are putting a lot of effort into developing software that will track membranes or microtubules in 3D," says McIntosh.

"While much has been accomplished, there is still room for improvement in image reconstruction and analysis," says Agard. "We are focusing our efforts on averaging objects within tomograms and image interpretation."

A look ahead

The initial applications of cryoET to answer biological questions have given way to a growing excitement in the cell biology community. In particular, as the technique continues to improve, scientists think it will soon be possible to obtain 3D structural maps of cell organelles or entire cells at sufficient resolution to locate within them 'molecular signatures' of individual macromolecules. Now, efforts are underway to develop a variety of methods, such as template matching and improved labels, that can be used in an automated process to locate these signatures. As cryoET resolution improves to the 2 nm range, it will be possible to fit high-resolution structures obtained by X-ray crystallography or other methods within the complete macromolecular context of the cell, leading to what Baumeister calls "pseudoatomic maps."

But while they anticipate these improvements, scientists in the field have much to be excited about right now. "The field is producing. At the moment people are getting data. There is a lot of space for results to be obtained even as we wait for advances," says Koster. "In 1980s most universities and hospitals had put away their EMs and they were gathering dust in the basement. Many scientists don't realize that in the last ten years microscopes have become fully automated. Now EM is a routine instrument," he adds.

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4. Trucco, A. *et al. Nat. Cell Biol.* **6,** 1071–1081 (2004).
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Suppliers guide: Companies that sell electron microscopes, specimen preparation equipment and detector technology

Company	Web address
Accelrys	http://www.accelrys.com/
Advanced Biotechnologies Inc.	http://abionline.com/
Alicona Imaging GmbH	http://www.alicona.com/
Able Software Corp.	http://www.ablesw.com/
Axon Instruments	http://www.moleculardevices.com/transition/
BAL-TECH	http://www.bal-tec.com/
BOC Edwards	http://www.bocedwards.com/
Campden Instruments Ltd.	http://www.campden-inst.com/
CamScan USA	http://camscan-usa.com/
Charles River Laboratories	http://www.criver.com/
CELLVIS	http://www.cellvis.de/english.html
Cressington Scientific Instruments Inc.	http://www.cressington.com/
Diatome U.S.	http://www.emsdiasum.com/diatome/
Digital Biomedical Imaging Systems AG	http://www.ditabis.de/
Delong Instruments	http://www.dicomps.com/
Denton Vacuum	http://www.dentonvacuum.com/
D'Outils Dumont SA	http://www.outils-dumont.com/
Electron Microscopy Sciences	http://www.emsdiasum.com/microscopy/
EBSciences	http://www.ebsciences.com/
EMITECH	http://www.emitech.co.uk/
Ernest F. Fullam Inc.	http://www.fullam.com/
FEI Company	http://www.feicompany.com
Fischione Instruments	http://www.fischione.com
Focus Electronics GmbH	http://www.focus-gmbh.com/
4pi	http://www.4pi.com/
Gatan Inc.	http://www.gatan.com
Hitachi High-Technologies America Inc.	http://www.hitachi-hta.com
JEOL	http://www.jeol.com
K.E. Developments	http://www.kedev.co.uk/
Leica Microsystems	http://www.em-preparation.com/website/sc_em.nsf
Marivac Canada Inc.	http://www3.ns.sympatico.ca/marivac/
M. E. Taylor Engineering Inc.	http://www.semsupplies.com/
Mercury Computer Systems Inc.	http://www.tgs.com/
Micro Star Technologies	http://www.microstartech.com/
Millbrook Scientific Instruments PLC	http://millbrook-instruments.com/
National A.T.E.	http://www.national-ate.com/
Numerical Algorithms Group	http://www.nag.co.uk/main_lifesciences.asp
Omicron NanoTechnology GmbH	http://www.omicron.de/
Optronics	http://www.optronics.com/
Oxford Instruments	http://oxinst.com
PARAGON Bioservices	http://www.paragonbioservices.com/
Photometrics (Roper Scientific Inc.)	http://www.prinst.com/
Polysciences Inc.	http://www.polysciences.com/shop/
Princeton Gamma-Tech	http://www.pgt.com/
Proscan	http://www.proscan.de/indexe.htm
ProSciTech	http://www.proscitech.com/
Quorum Technologies	http://www.quorumtech.com/
RMC Products	http://www.rmcproducts.com/
SemTech Solutions Inc.	http://www.semtechsolutions.com/
Sidec Technologies	http://www.sidectech.com
Soft Imaging System	http://www.soft-imaging.com/
South Bay Technology Inc.	http://www.southbaytech.com/
Structure Probe Inc.	http://www.2spi.com/
Ted Pella Inc.	http://www.tedpella.com/
Tousimis	http://www.tousimis.com/EM_products.html
Tietz Video and Image Processing Systems	http://www.tvips.com/
Vibratome	http://www.vibratome.com
Visitec	http://www.visitec-em.de/
Zeiss	http://www.smt.zeiss.com

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