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Cryo-electron tomography of bacteria: progress, challenges and future prospects

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Abstract

Recent advances in three-dimensional electron microscopy provide remarkable tools to image the interior of bacterial cells. Glimpses of cells at resolutions that are 1–2 orders of magnitude higher than those currently attained with light microscopy can now be obtained with cryo-electron tomography, especially when used in combination with new tools for image averaging. This Review highlights recent advances in this area and provides an assessment of the general applicability, current limitations and type of structural information that can be obtained about the organization of intact cells using tomography. Possible future directions for whole cell imaging are also discussed.

The visualization challenge

Deciphering the structure, function and spatial organization of multi-component molecular machines inside the fluid architecture of cells is an emerging frontier in cell biology. However, the importance of this problem has been recognized for a long time. Nearly two decades ago, Goodsell, who popularized scale drawings of cells and viruses in numerous scientific journals, made the following statement¹: “A clear picture of the interior of a living cell that shows the average distribution of molecules at the proper scale, the proper concentration and with no missing parts, seems to me to be central to the understanding of the workings of life. However, this type of picture is virtually absent from the popular literature. There is no single experimental method to determine the information needed for their construction. Electron microscopy gives a view that is too coarse: subcellular structure is studied but the individual molecules are not seen”.

The problem that Goodsell identified is at the heart of modern cell biology and reflects the growing interest in deciphering the connection between structure and function in the context of an intact cell. Fortunately, Goodsell’s categorization of electron microscopy as a tool incapable of providing a view of individual molecules in cells is no longer valid. Methods in three-dimensional (3D) electron microscopy such as electron tomography^{2–4} now provide powerful new tools to bridge the gap in knowledge between cellular architecture as revealed

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SUPPLEMENTARY INFORMATION

See online article: S1 (figure) | S2 (figure)

by light microscopy, and the molecular structures of individual protein assemblies studied by X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and cryo-electron microscopy^{5–9}. These new imaging technologies, coupled with powerful genetic tools for the manipulation of cell function and computational tools for the analysis of complex data, provide new and exciting opportunities to understand bacterial physiology at a molecular level.

Despite the great potential of electron tomography, many fundamental questions in structural microbiology also currently lie beyond its reach. Useful images cannot be obtained at present from many commonly studied bacteria, and for those bacteria that can be analysed, there is considerable variation in the quality of information that can be obtained from different regions of the cell and from different types of macromolecular complexes in the cell. Yet, in at least some cases, molecular resolution can be attained and, with the use of advanced methods for image averaging, structure and conformational changes in specific membrane proteins can be described in the context of the native cell membrane. The Review highlights recent successes of bacterial electron tomography and assesses the unique features of the bacteria that have been most amenable to this technology. Challenges that lie ahead are described and an overview of other emerging technologies that may become available in the near future for high-resolution imaging of whole bacterial cells is provided.

Introduction to electron tomography

The principles underlying 3D imaging of a whole bacterial cell by electron tomography are the same as those developed decades ago for computerized axial tomography. A series of projection images are recorded from the specimen by varying its orientation relative to the incident electron beam. Each of these images contains information about the 3D structure of the cell collapsed onto a single plane, and the actual 3D arrangement of components in the specimen can be recovered by computationally combining the information in the images. FIG. 1 outlines the practical steps that precede the generation of a 3D image of a single cell by electron tomography.

The first and crucial step in specimen preparation is ‘vitrification’ of the cell suspension by rapidly plunging a thin film of the bacterial culture into a cryogen such as liquid ethane that is cooled by liquid nitrogen to temperatures of ~ 100 K. The bacterial suspension is typically deposited on an electron microscope grid covered by a carbon film with holes; bacteria that end up in a hole are embedded in a thin layer of ice and can be imaged without interference from the carbon film. The speed of plunge-freezing is such that it results in the formation of vitreous ice capturing the cells in their native state at the instant of freezing. Although there have been improvements in automation of plunge-freeze techniques over the years^{10,11}, the original ideas and methods pioneered by Dubochet and colleagues over two decades ago¹² have remained largely unchanged. The frozen grid is loaded at liquid nitrogen temperature onto a specimen holder that is then transferred onto the specimen stage of the microscope, which is maintained throughout the imaging process at temperatures of ~ 100 K or lower.

Almost all current bacterial tomography uses microscope specimen stages that are cooled by liquid nitrogen. Commercial instruments with specimen stages that are cooled by liquid

helium and which can operate at temperatures below 10 K are also available. Although this capacity has been available for many years, advances in cryo-electron tomography to date have come with the use of instruments that operate at liquid nitrogen temperatures. Initial studies assessing cryogen performance suggest that the tomographic data obtained at helium temperatures may not be better than that collected at liquid nitrogen temperatures¹³, but it is conceivable that the methods needed to obtain data of similar or better quality at liquid helium temperatures remain to be developed. Over the past decade, automation of tomographic data collection has arguably been the most important advance in the development of hardware for electron microscopy. Automation allows great precision and reproducibility in data acquisition and is perhaps the single biggest factor that has led to the increase in tomography of prokaryotic and eukaryotic cells¹⁴ alike.

There are numerous strategies for converting the series of images obtained at different tilts from an electron microscope into a 3D volume that is referred to as a 'tomogram'. The underlying principle is to carry out the inverse of the process that generates each projection image by re-projecting the image back along the direction of the projection. Electron microscopes record projection images, and each image contains information from all heights of the specimen collapsed onto a single plane. However, by recording a series of images in which the orientation of the specimen is varied relative to the incident beam over a wide angular range, information required to describe the 3D structure of the imaged object can be obtained. The density of each image in the tilt series is first smeared back evenly along the direction of the electron beam into an imaginary box, the height of which is approximately equal to the thickness of the specimen. This procedure is repeated for each of the tilted images. When these serial back projection profiles are combined, the spatial distribution of density in the superimposed reconstruction is proportional to the original density of the object. Programs that implement these methods are available both as commercial packages and in freely distributed academic software packages such as **IMOD**¹⁵ or those based on the package **ImageJ** developed at the National Institutes of Health (NIH), USA^{16,17}.

The raw tomograms contain information from all regions of the specimen that scatter the incident electrons, including regions inside and outside the cell. The images and tomograms are generally rather noisy, in part because of the need to use low electron doses in order to minimize radiation damage to the specimen during data collection. To improve image contrast, tomograms are often 'denoised' to facilitate object recognition by suppressing higher frequencies in the image. Following denoising, tomograms can be 'segmented' to highlight selected regions of the volume such as ribosomes, membranes or filaments using manual or automated procedures to appreciate the broad organizational features of the cell. Data interpretation by segmentation is largely qualitative, but efforts to obtain a better assessment of the fidelity of recovering the information in the images using algorithms such as non-linear anisotropic diffusion are underway^{18,19}.

Imaging intact bacterial architecture

Cryo-electron tomography has enabled determination of the 3D architectures of several types of intact bacterial cells in the frozen state. Rapid freezing of thin aqueous films of bacterial cells, typically at rates exceeding 10^5 K per second¹², leads to rapid vitrification,

which therefore preserves the spatial arrangement of cellular components present at the moment of freezing. Imaging these intact frozen cells under conditions that minimize radiation-induced damage (that is, preserving structure close to the native physiological state) has yielded insights into the internal organization of many commonly studied Gram-negative bacteria, archaeobacteria, cyanobacteria, mycobacteria and mycoplasma cells^{20–35}. Gram-positive bacteria are a notable exception, in part because the peptidoglycan layer³⁶ is a significant addition to the overall thickness of the cell, making acquisition of high-quality transmission electron microscopic images through the cell difficult. In TABLE 1, we list published examples (up to 2008) of whole bacterial cells that have been imaged by cryo-electron tomography (see Supplementary information S1 (figure)), and in FIG. 2, we show selected images from a few of these publications that capture key highlights of the work. Several common themes have emerged from the analyses so far, as discussed in the following section.

Readily detectable subcellular features

The most distinct and easily resolved features by cryo-electron tomography are membranes at the peripheries of the cells, such as inner and outer membranes in Gram-negative bacteria^{20,37,38}, the S-layer in certain bacteria or archaea^{24,39}, and the peptidoglycan layer in Gram-negative bacteria (TABLE 1). Internal membranes such as those incorporating specialized components in the photosynthetic bacterium *Rhodospseudomonas viridis* are also easily resolved³¹, and in certain cases, the resolution has been adequate to observe the 4 nm spacing between the two leaflets of the lipid bilayer³⁵, which provides a measure of the types of features that can be resolved.

Particles with strong contrast, that have dimensions consistent with the sizes and shapes of ribosomes, have also been seen in lysed cells⁴⁰ and in virtually all of the intact cells that have been imaged by cryo-electron tomography. In a few instances, analyses have been taken beyond qualitative observation in attempts to statistically quantify the entire complement of ribosomes present in cellular tomograms^{19,23,41}. The nucleoid area is visualized perhaps most clearly in the highly packed genome of the predatory bacterium *Bdellovibrio bacteriovorus*²⁰ but is also evident in other cell types (see Supplementary information S1 (figure)) as a dispersed central region that excludes ribosomes. Filaments in the cytoplasm^{20,21,42}, the periplasm²² and even on the surface of gliding bacteria³² have been visualized, although their molecular composition remains controversial or unknown in most cases.

Unusual snapshots, such as the unexpected image capture of a virus that was found attached to a small archaeal cell²³ or trapped magnetite particles in invaginated cellular membranes^{30,43} have also been documented. Other features that have been commonly observed include chemoreceptor arrays at the cell pole^{35,44,45}, phosphorus-rich cytoplasmic bodies, the elemental compositions of which have been verified in some instances by electron spectroscopic imaging^{20,46}, carboxysomes³⁴, which are storage sites for the ubiquitous Rubisco enzymes, the rotor complex, and other components of the bacterial flagellum^{20,38}.

Detecting smaller complexes

Despite the success in the imaging of membranes, filaments, ribosomes and some prominent assemblies, it is fair to say that the vast majority of cellular components have yet to be visualized by cryo-electron tomography. During phase-contrast imaging of thin specimens by cryo-electron microscopy, the specimen is deliberately defocused to generate contrast from all regions that diffract electrons, this then allows specific components to be identified by the differences in their electron scattering and that of their surroundings. Even in the best case, this contrast is weak because low electron doses are used in cryo-electron microscopy to minimize radiation damage⁴⁷. However, as nucleic acids scatter electrons differently because of their different elemental composition, ribosomes stand out easily against a largely protein-only background. Similarly, membranes and filaments are easily distinguished because of their extended nature and their higher local scattering profiles as compared with the surrounding milieu. Chemoreceptors are clustered in an identifiable region of the cell³⁵, and have long cytoplasmic domains, making their identification relatively easy and unambiguous. However, most proteins generate similar scattering profiles and, because they rarely show macroscopic segregation, do not stand out against the background. One way to obtain additional information is to use procedures such as template matching⁴⁸ in which the 3D volume is searched against a low resolution template of structures that are expected to be present such as the bacterial protein GroEL. Such methods have already been shown to have promise in the imaging of synthetic, reconstituted vesicles in which there are clear backgrounds with only buffer⁴⁹. However, they are not likely to be useful to detect proteins in the complex milieu of a cell unless there is some unique chemical feature of the protein in question that lends itself to higher contrast than those in its immediate vicinity, or there are specialized circumstances such as partially lysed cells, in which selected components can be visualized at higher signal-to-noise ratios than is possible in intact cells.

Mimicking the success that labelling methods (for example with green fluorescent protein or similar moieties) have had in the detection of smaller complexes by optical microscopy, attempts have been made to tag proteins with electron-dense moieties to improve contrast for electron tomography. For example, the addition of metallothionein sequences to proteins enables the binding of heavy metals that could provide contrast^{50,51}. The bound metals could potentially be detected simply by virtue of the higher contrast that would result from their higher atomic number, or by spectroscopic imaging methods that detect atom-specific signatures^{52,53}, although these latter methods could need the use of significantly higher electron doses. Photosensitive arsenic compounds such as ReASH⁵⁴, which can be used for site-specific protein labelling in both fluorescence and electron microscopic imaging, have been useful for localizing membrane proteins and studying organellar architecture in mammalian cells and could well be applied to the study of bacterial morphology. Additional strategies for the detection of smaller structures are based on correlative imaging methods^{55,56} that allow the use of fluorescence imaging to locate a particular region of interest in the cell for targeted electron tomographic imaging, which could provide both a better spatial context and a way to restrict the search for molecular signatures to a small region of the cell. All of these technologies are still in an early phase and will need considerable development before their routine use can be advocated.

The use of labelling methods may be useful not just for electron tomography, but for a complementary approach that uses soft X-rays to obtain 3D images of frozen cells at resolutions that may exceed 100 nm in the near future. Although initial experiments were carried out using synchrotron sources, which guarantee a high photon flux⁵⁷, there is now an increase in the availability of simpler laboratory radiation sources⁵⁸ and tabletop sources^{59–61} that can provide in-house 3D cellular imaging. Despite the modest resolutions attained with these methods, they may still be valuable for protein localization by tagging proteins with X-ray opaque probes such as gold and using detection strategies based either on simple absorption or using specific absorption edges of atoms such as titanium (TiO₂ nanoparticles) for differential imaging⁶².

The importance of size

One common theme that emerges from a survey of bacteria that are amenable to analysis by electron tomography (TABLE 1) is that width is by far the most important factor determining success. At the intermediate voltages (300 kV) currently used for cryo-electron tomography, the mean free path (that is, the distance travelled between two scattering events) of an electron through the specimen is ~ 350 nm⁶³. This roughly corresponds to the average width of the slimmest bacteria such as *Bdellovibrio bacteriovorus* and *Caulobacter crescentus*, the internal cellular contents of which can be visualized with the highest contrast. Bacteria such as *Flavobacterium johnsoniae* (see Supplementary information S1 (figure)) are slightly wider (~ 400 nm), and bacteria such as *Escherichia coli* can be even wider (~ 1000 nm). At these larger widths, cellular components with high local contrast, such as the photosynthetic membranes in *Rhodospseudomonas viridis*, can still be visualized³¹, but even well-defined complexes such as ribosomes can be hard to locate reliably. Nevertheless, polar regions of the cells can still be analysed, and much can be learnt about protein complexes such as chemoreceptor assemblies in this region of the cell³⁵. Therefore, the simple answer to why only a handful of proteins can be presently visualized in whole cells is that, unless there is something different about their composition that results in a significantly different scattering profile, there is not enough contrast to locate them. Finding a means to enhance protein visibility in electron tomographic data is clearly an important area for future development.

A complementary approach to imaging intact cells by electron tomography is to eliminate the challenge of examining thick cells by dividing the cell into sections. Although this approach does not enhance contrast, it does offer significant advantages for imaging cellular components that are otherwise poorly visualized in intact cells, as discussed below.

Strategies for sectioning frozen cells

Collecting images that are useful for tomographic reconstruction requires transmission of electrons through the specimen with minimal inelastic scattering. This sets ~ 0.5 mm as the upper limit for specimen thickness at which images have enough contrast so that subcellular structures can be detected with state-of-the-art microscopes. A thickness of ~ 0.5 mm at zero degrees tilt corresponds to a thickness of ~ 1 mm at 60 degrees tilt. Internal membranes, and structures at the thinner edges of the cell, can still be somewhat discerned from tomograms

obtained from bacteria as thick as ~ 1 mm, but minimal information is gained about the structure of the rest of the cell. Historically, cells and tissues have been investigated by imaging sections prepared in a microtome to overcome this limitation (FIG. 3a).

Cells that have been frozen rapidly at high pressure to prevent damage from ice crystal formation can be sectioned at ultra-low temperatures into slices without loss of the ‘vitrified’ state of the cellular material. Sections can be imaged at liquid nitrogen temperatures to obtain projection images. Alternatively, a series of tilted images can be collected and combined to obtain a representation of the 3D volume using electron tomography. Therefore, one method to determine the 3D architecture of thicker cells is to section a small pellet of frozen cells into a series of ribbons. Each of these sections is thin enough to be imaged by transmission electron microscopy, and the individual 2D images or section tomograms can be combined together to generate a tomogram of the larger cell. This procedure, referred to sometimes as CEMOVIS (cryo-electron microscopy of vitrified sections) is a promising approach not just for 3D imaging, but also to reveal specific aspects of cellular structure such as lipid membranes or cytoplasmic filaments (TABLE 2).

Ion-abrasion scanning electron microscopy is yet another complementary strategy to image large cells and tissue. It uses a focused beam of gallium ions to section through the material by ion abrasion (FIG. 3b) and the newly created surface can then be imaged with a scanning electron beam. Iterating this procedure builds up the 3D structure one surface at a time. The validity of this approach has been successfully demonstrated for routine 3D imaging of plastic-embedded specimens at ~ 6 nm in-plane resolution⁶⁴, but the application to frozen specimens is still in an early phase of development⁶⁵. The use of the focused ion beam is not restricted to being coupled with scanning electron beam imaging; it can also be used to reduce the thickness of frozen thicker specimens to enable imaging by cryo-electron tomography⁶⁶.

Insights from imaging thin sections

Advances in the technology for rapid freezing and the use of microtomes that can operate at temperatures of –150 °C or below have allowed the preparation of thin sections from frozen bacterial cells with thicknesses as small as 20 nm (TABLE 2). Selected examples of images obtained from these cryo-sections are shown in FIG. 4, with a more complete collection presented in the Supplementary information S2 (figure). In comparison to images from whole cells, internal regions of cells such as membranes and ribosomes can be seen with far higher contrast in these sections. Images from vitreous sections have been particularly useful to resolve fundamental structures, such as a periplasmic zone in Gram-positive bacteria^{67,68}, and to provide insights into the precise organization of membranes in a range of bacteria^{26,36,67–73}. Structural questions such as the packing arrangement in the nucleoid region⁷⁴, and the localization of cytoplasmic filaments involved in plasmid DNA segregation⁷⁵ have been easier to address using thin sections where the components can be visualized with much better contrast in the absence of interference from the depth of the whole cell. It is important to note, however, that although the imaging of vitreous sections can provide higher contrast, the entities that are visualized are the same as those that have also been observed in whole cells, albeit at a somewhat lower contrast.

In general, there are four strategies for the preparation and imaging of sections in an electron microscope. The oldest approach involves the use of an aldehyde (typically glutaraldehyde) to crosslink cellular components, followed by treatment with osmium-containing reagents for further fixation and staining of certain components and embedment in a plastic resin that encases the fixed stained cells. The resin blocks are mounted in a microtome, and sections with thicknesses ranging from 20 nm to as much as 1 mm can be prepared and imaged by electron microscopy. A gentler approach to fixation requires high-pressure freezing^{76,77} followed by solvent exchange and staining when the sample is still frozen (freeze substitution). A third approach used for immunolabelling is to chemically fix the cells with glutaraldehyde followed by freezing and sectioning at -100°C , and application of antibodies after the sections are picked up on the surface of an aqueous phase⁷⁸. The strategy that leads to the highest level of preservation is that in which sections are prepared at temperatures below -150°C from vitrified specimens (FIG. 4; TABLE 2).

In cryo-electron microscopic images, the contrast derives from intrinsic scattering of electrons, so that the information present in the image is not altered by either fixation or the use of heavy metal staining procedures that involve dehydration and usually result in selective staining of cellular components. Although there is a widely held view that this makes cryo-electron microscopy superior to room temperature electron microscopy, this is not always necessarily the case and depends on the type of question being addressed. Fixed stained specimens do not provide useful protein structural information at resolutions much better than $\sim 100 \text{ \AA}$. However, it is also the case that unless cryo-electron microscopy is performed using relatively low electron doses, beam damage can give rise to artefacts that are not readily apparent on qualitative inspection of individual tomograms. Beam damage is also an important factor for stained specimens, and there can also be significant mass loss during the course of collecting data from stained, plastic-embedded specimens.

There is little evidence that locations of key macromolecular complexes in the bacterial cytoplasm are significantly rearranged as a result of fixation and/or staining, and, in fact, comparative analysis with images from intact bacteria suggests that molecular structure at least of cytoplasmic filaments is generally maintained in high pressure frozen, freeze-substituted specimens⁷⁹. In chemically fixed cells, damage to structure can arise from glutaraldehyde crosslinking, treatment with osmium and from the use of higher electron doses. In principle, all three factors can contribute to distortions, but a careful comparison of vitreous section imaging from fixed cells either with or without subsequent osmium treatment, establishes that the damage from fixation itself is minimal whereas osmium treatment can lead to alterations in ultrastructure⁷³. Although these observations do not completely eliminate concerns arising from the effects of fixation itself, it is also true that many important advances have come with the imaging of plastic-embedded sections, in which sacrificing fine structure has resulted in other gains. Therefore, higher doses in combination with energy dispersive X-ray analysis were used to map the elemental composition of cellular compartments in anammox bacteria⁸⁰ and to gain an understanding of the potential role of iron in energy generation. Tomography of plastic sections has also been useful in delineating 3D membrane connectivity in diverse instances^{81,82}.

Molecular resolution by image averaging

Although a key focus of efforts to image whole cells and thin sections by electron tomography has been to describe subcellular architecture, information in the tomograms could potentially be used to derive molecular structures of cellular components. The structures of proteins and protein complexes have been determined exclusively under conditions in which the proteins have been purified away from their native context, and often truncated at various regions to facilitate structure determination by X-ray crystallography. This raises questions about the relevance of structures determined by X-ray crystallography and NMR spectroscopy that could be addressed if it were possible not just to locate these complexes in the cell, but to actually determine the 3D structure of proteins and complexes when they are still present in intact bacterial cells.

How feasible is this approach? One challenging aspect of the problem is that the signal-to-noise ratios in electron tomograms are generally far too low to reveal anything meaningful about the 3D structures of individual protein complexes. However, if the locations of specific protein complexes can be identified unambiguously, it should be possible to carry out 3D averaging of hundreds or thousands of individual subvolumes to improve the signal-to-noise ratios. The idea of using image-averaging approaches to derive 3D structures of complex macromolecular assemblies is of course not new, and has been a central idea in low-dose biological electron microscopy of purified protein complexes for over two decades⁶. At the heart of these methods is the assumption that the entities being averaged are chemically and structurally homogeneous at the resolution of interest. The same conceptual approach can also be applied to 3D shapes in reconstructed tomograms, but there are some additional challenges that need to be considered. In general, it is impossible to know *a priori* the extent of conformational heterogeneity of the various copies of the protein complex of interest present in the milieu of the cell. If the individual 3D images in the collection are generated from distinct 3D conformations, a further level of classification is required to identify and separately average the individual conformations. One further problem arises from the limited range of tilt inherent to electron tomography, which results in modification of the reconstructed shapes of individual complexes by the ‘missing wedge’⁸³. The presence of the missing wedge effectively results in the differences in appearance of the same object depending on their relative orientation with respect to the beam. Significant advances have been made recently^{84,85} by devising rigorous computational methods to overcome these problems, therefore, paving the way forward for analysing molecular structures of macromolecular complexes in the cell.

FIG. 5 highlights examples in which the use of tomography combined with 3D image averaging has been used to derive structural information on specific protein assemblies. Oritz *et al.*⁸⁶ reported a ribosomal structure derived from averaging ~ 236 individual ribosome subvolumes identified in tomograms of intact *Spiroplasma melliferum* cells, and suggested that the orientations of ribosomes in the cell could be identified. Murphy *et al.*³⁸ averaged 20 individual subvolumes of the flagellar motor from cells of *Treponema primitia* to obtain a 3D structure of the arrangement of subunits in an intact cell. Whereas ribosomes and the flagellar motor are arguably unique, and unrepresentative of the average protein in a cell, three recent reports^{44,45,87} indicate the broader potential of these averaging approaches

to analyse the structures of membrane protein assemblies (FIG. 5) in bacterial cells, at least in instances when they are clustered in the cell membrane. Analysis of the chemotaxis signalling complex in *Caulobacter crescentus* revealed the partially ordered, hexagonally packed arrangement of chemoreceptors near the pole of the cell, as well as the relative arrangement of receptors and associated signalling proteins⁴⁵. Classification and averaging of individual subvolumes corresponding to individual chemoreceptors overproduced in *E. coli* has led to determination of the 3D structure of the intact chemoreceptor for serine (Tsr), and is also the first report of the 3D structure of a full-length chemoreceptor at any resolution. The advance represented by this work is that not only was it possible to determine the averaged 3D structure of the receptor in the membranes of the cell at resolutions of ~ 30 Å, but two discrete coexisting conformations of the receptor could be separated⁸⁷. The functional relevance of these conformations was established by changing the relative proportions of the two conformations by adding ligands known to bind the receptor and influence receptor activation.

The demonstration that 3D structure and structural changes can be studied in the context of an intact cell heralds a new era in the use of electron tomography combined with 3D image averaging. There is reason to hope that this strategy can be applied to other membrane proteins, especially in the context of over-produced recombinant proteins as this could allow the protein of interest to be more easily distinguished and located in the cell. Further, the use of correlative microscopy and/or the use of genetically tagged proteins could be useful to identify regions of the cell where the protein of interest is present at high concentrations.

Concluding remarks

3D electron microscopy has come a long way in the past few years and is now increasingly perceived as a technology that can contribute significantly to study structural aspects of intact bacterial cells. The focus of this Review has been to explore the instances in which cryo-electron tomography of intact cells has been successful, and to provide a perspective on the origins of this success. Given fundamental technical limitations of electron tomography of whole cells, it is likely that for many years to come we will continue to learn about bacterial architecture of whole cells largely through tomographic analyses of the smaller bacteria with widths less than ~ 0.5 μ m. Undoubtedly progress will also be made on understanding structural aspects of larger bacteria as procedures for the generation of vitreous sections become more streamlined. Tools for 3D protein localization using electron-dense tags may also revolutionize prospects of mapping protein localization at the nanoscale, but these methods are at an early stage of development. For thicker bacteria, the use of high pressure freezing followed by freeze substitution could be an excellent strategy to obtain 3D reconstructions of the interior of the cell by imaging plastic-embedded sections at room temperature. The use of imaging at high electron doses under non-cryogenic conditions may have an advantage as the use of stains with different chemical compositions could enable visualization of components that display little contrast difference in cryogenic conditions. Chemical imaging using electron microscopy, and also with powerful new optical technologies such as those based on Raman scattering microscopy⁸⁸ have great promise as we extend the envelope of imaging from probing architecture to unravelling chemistry. It is

safe to predict that the exponential growth of this field will continue unabated in the coming decade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 | From cell to tomogram.

Schematic diagram illustrating the sequence of steps involved in obtaining a three-dimensional image of a plunge-frozen cell using cryo-electron tomography. **a** | A small volume (typically $\sim 3\text{--}5\ \mu\text{l}$) of a suspension of cells in culture is deposited on a holey carbon grid and then plunge-frozen in liquid ethane cooled to temperatures of $\sim 100\ \text{K}$ by liquid nitrogen. **b** | The grid is then transferred into the column of an electron microscope for collection of a series of images at varying tilts. **c** | The series of tilted images is then converted to a three-dimensional volume (tomogram) that provides a representation of the distribution of densities of cellular components.

Figure 2 | Selected examples of findings from cryo-electron tomography of bacterial cells. (see TABLE 1 and Supplementary information S1 (figure) for complementary information). **a** | 19 nm tomographic slice from the tomogram of a *Caulobacter crescentus* cell highlighting multiple features including filaments adjacent to the inner membrane (dashed white box). Scale bar 200 nm. **b** | 5 nm tomographic slice from a wild-type *Escherichia coli* cell showing the polar region of the cell and delineation of inner and outer membranes which contain a chemotaxis receptor array composed of chemoreceptor, CheA and CheW molecules. Scale bar 50 nm. **c** | 1.8 nm longitudinal slice from tomogram of a *Borrelia burgdorferi* cell highlighting a ribbon of nine periplasmic flagella that forms a right-handed arch in the cytoplasm at the bottom of the cell, as viewed from the top. Scale bar 200 nm. **d** | *x-y* tomographic slice from tomogram of a *Mycobacterium bovis* bacille Calmette-Guérin cell illustrating the cytoplasmic membrane (CM), and the mycobacterial outer membrane (MOM). Scale bar 50 nm. **e** | Schematic view of a bacterial cell modelled on electron tomographic analysis of the Gram-negative bacterium *Bdellovibrio bacteriovorus*²⁰. The inset highlights insights gained into bacterial architecture such as the precise organization of

membranes, the arrangement of the nucleoid, the distribution of cytoplasmic filaments as well as the three dimensional structures of a large number of protein complexes in the membrane and the cytoplasm. GF, gold fiducial for image alignment; IM, inner membrane; OM, outer membrane; Phb, putative poly- β -hydroxybutyrate granule; PG, peptidoglycan layer; Rib, probable ribosome; SL, surface layer; St, stalk. Part **a** is reproduced, with permission, from REF. 21 © (2006) Blackwell Scientific publications. Part **b** is modified, with permission, from REF. 35 © (2007) National Academy of Sciences. Part **c** is reproduced, with permission, from REF. 22 © (2009) American Society for Microbiology. Part **d** is reproduced, with permission, from REF. 26 © (2008) National Academy of Sciences.

Figure 3 | Two alternative strategies for three-dimensional reconstruction of thick cells.

Cells that are too thick for imaging in the intact state can be first vitrified by freezing rapidly at high pressures, and then sectioned in a microtome. **a** | A ribbon of serial sections is transferred to an electron microscopic grid, imaged to obtain either a series of 2D or 3D images that can then be stacked together to obtain a 3D reconstruction of the entire cell. **b** | Alternatively, frozen cells can be progressively abraded using ion-abrasion scanning electron microscopy, and a 3D representation of the cell can be obtained by stacking together the series of images of each newly created surface. Figure reproduced, with permission, from REF. 64 © (2007) Academic Press.

Figure 4 |. Selected findings from cryo-electron microscopy of vitreous sections of high-pressure frozen bacterial cells.

a | Cryo-electron microscopy image of a vitreous section of the cyanobacterium *Lyngbya majuscula* cell envelope identifying the peptidoglycan layer (P), outer membrane (OM), putative S-layer (S), cytoplasmic membrane (CM) and several other additional layers (labels) and zones of the extracellular matrix containing 4 nm filaments (arrowhead) (scale bar 100 nm). **b** | 1.8 nm tomographic slice of frozen-hydrated cryo-section of a high-pressure frozen *Prochlorococcus* MIT9313 cell showing intracytoplasmic lamellae (label) connected by a membrane-lined channel (scale bar 100 nm). **c** | Projection image of vitreous section from a high-pressure frozen *Escherichia coli* cell engineered to overproduce the serine chemoreceptor. The two leaflets of the OM and of the inner membrane (IM) as well as the P are visible, as is the invaginated IM (iIM), a consequence of receptor overproduction (scale bar 50 nm). **d** | End-on view of filaments of the bacterial analogue ParM contained within a vitreous cryosection of an *E. coli* cell expressing high levels of ParM using a T7-driven promoter on a high-copy number plasmid (scale bar 10 nm). Part **a** is reproduced, with permission, from REF. 89 © (2004) Oxford University Press. Part **b** is reproduced, with permission, from REF. 34 © (2007) American Society for Microbiology. Part **c** is reproduced, with permission, from REF. 72 © (2004) Blackwell Scientific Publications. Part **d** is reproduced, with permission, from REF. 75 © (2008) American Assn for the Advancement of Science.

Figure 5 | Molecular structures of bacterial components *in situ* determined by three-dimensional averaging of individual volumes extracted from whole cell tomograms.

a | Visualization of the three-dimensional structure (3D) and partially ordered hexagonal arrangement of chemoreceptor arrays in *Caulobacter crescentus* cells. **b** | The two predominant receptor conformations of the *Escherichia coli* serine receptor Tsr derived by 3D averaging, corresponding to the 'kinase-off' and 'kinase-on' signalling states. **c** | The flagellar motor in cells of the *Treponema primitia* (scale bar 20 nm). **d** | Distribution and structure of ribosomes (indicated by the arrow) in *Spiroplasma melliferum* with green and yellow indicating higher or lower levels of accuracy of detection (scale bar 100 nm). Part **a** is reproduced, with permission, from REF. 45 © (2008) American Society for Microbiology. Part **b** is reproduced, with permission, from REF. 87 © (2008) National Academy of Sciences. Part **c** is reproduced, with permission, from [Nature](#) REF. 38 © (2006) Macmillan Publishers Ltd. All rights reserved. Part **d** is reproduced, with permission, from REF. 86 © (2006) Academic Press.

Cryo-electron tomography of intact bacterial cells

Table 1 |

Cell type	Main features identified	Microscope location	Year	Refs
Archaea				
<i>Pyrobaculum aerophilum</i> , <i>Sulfolobus</i> spp	S-layer	Martinsried, Germany	1998	24
<i>Pyrodicticum cannulae</i>	Cannulae, vesicles	Martinsried, Germany	2003	33,39
ARMAN	Tubular internal structures	Berkeley, USA	2008	23
<i>Nanoarchaeum equitans</i>	Cytoplasmic fibres	Martinsried, Germany	2008	29
Gram-negative bacteria (rod-shaped)				
<i>Bdellovibrio bacteriovorus</i>	Nucleoid, cytoplasmic filaments	NCI/NIH, USA	2008	20
<i>Caulobacter crescentus</i>	Cytoplasmic filaments, chemotaxis receptors, dense granules	Caltech, (USA; NCI/NIH, USA; Berkeley, USA	2006–2008	21,45, 42,46
<i>Escherichia coli</i>	Chemotaxis receptors	NCI/NIH, USA	2007, 2008	35,87
<i>Flavobacterium johnsoniae</i>	Surface filaments	NCI/NIH, USA	2007	32
<i>Rhodospseudomonas viridis</i>	Photosynthetic membranes	Weizmann, Israel	2008	31
<i>Magnetospirillum magneticum</i> and <i>Magnetospirillum gryphiswaldense</i> (magnetotactic bacteria)	Magnetosomes	Caltech, USA; Martinsried, Germany	2006	30,43
Gram-negative bacteria (spirochetes)				
<i>Spiroplasma melliferum</i>	Cytoplasmic filaments, ribosome distribution	Martinsried, Germany	2005, 2006	37,86
<i>Treponema primitia</i>	Flagellar motor, cell surface components	Caltech, USA	2006, 2008	28,38
<i>Treponema denticola</i>	Periplasmic and cytoplasmic filaments	Albany, USA	2008	27
<i>Borrelia burgdorferi</i>	Periplasmic filaments	Albany, USA	2008	22
Miscellaneous				
<i>Prochlorococcus</i> spp (cyanobacterium)	Photosynthetic lamellae	Albany, USA	2007	34
<i>Mycobacterium bovis</i> bacille Calmette-Guérin and <i>Mycobacterium smegmatis</i>	Membrane bilayer structure	Martinsried, Germany	2008	26
<i>Mycoplasma pneumoniae</i>	Attachment organelle	Heidelberg, Germany; Caltech, USA	2006	25,41

ARMAN, archaeal Richmond Mine acidophilic nanoorganisms; NCI, National Cancer Institute; NIH, National Institutes of Health.

Table 2 |

Cryo-electron microscopy of sections from intact, vitrified bacterial cells

cell type	Main features identified	Laboratory location	Year	Refs
Gram-negative bacteria				
<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>	Membrane distribution and peptidoglycan layer	Lausanne, Switzerland	2003	69
<i>E. coli</i>	Lipid bilayer leaflets, chemotaxis receptors	NCI/NIH, USA	2004	72
	Filaments	Cambridge, UK	2008	75
Gram-positive bacteria				
<i>Bacillus subtilis</i>	Delineation of peptidoglycan, periplasm	Lausanne, Switzerland	2005	36
<i>Staphylococcus aureus</i>	Cell division	Guelph, Canada	2006, 2007	67, 70
<i>Streptococcus gordonii</i> , <i>Enterococcus gallinarum</i>	Septum in dividing cells	Lausanne, Switzerland	2006	68
Mycobacterial family				
<i>Mycobacterium smegmatis</i> , <i>Mycobacterium bovis</i> bacille Calmette-Guérin, <i>Corynebacterium glutamicum</i>	Inner and outer membrane bilayer profiles	Lausanne, Switzerland; Martinsried, Germany	2008	26, 73
Cyanobacteria				
<i>Lyngbya majuscula</i>	Membranes and S-layer	Lausanne, Switzerland	2004	89
<i>Prochlorococcus</i> spp.	Photosynthetic membrane arrangement	Albany, USA	2008	34
Radiation-resistant bacteria				
<i>Deinococcus radiodurans</i>	Nucleoid region	Lausanne, Switzerland	2005, 2006	74, 91

NCI, National Cancer Institute; NIH, National Institutes of Health.