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Electron cryo-tomography captures macromolecular complexes in native environments

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Transmission electron microscopy has a long history in cellular biology. Fixed and stained samples have been used for cellular imaging for over 50 years, but suffer from sample preparation induced artifacts. Electron cryo-tomography (cryoET) instead uses frozen-hydrated samples, without chemical modification, to determine the structure of macromolecular complexes in their native environment. Recent developments in electron microscopes and associated technologies have greatly expanded our ability to visualize cellular features and determine the structures of macromolecular complexes *in situ*. This review highlights the technological improvements and the new areas of biology these advances have made accessible. We discuss the potential of cryoET to reveal novel and significant biological information on the nanometer or subnanometer scale, and directions for further work.

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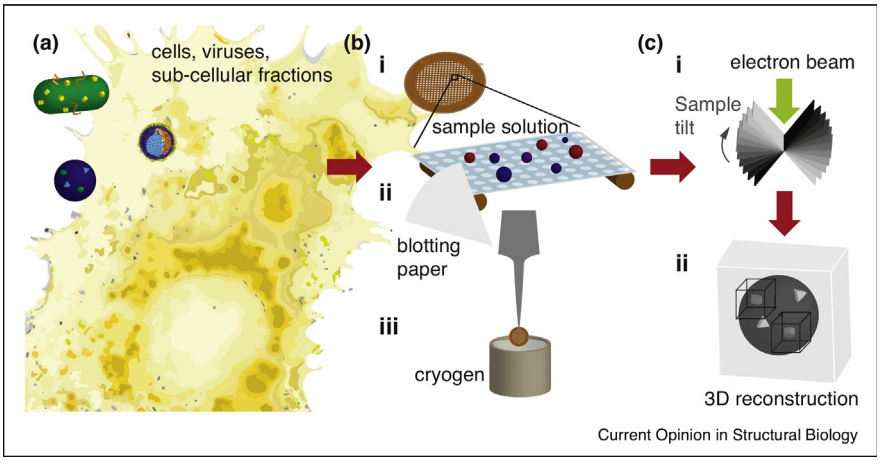
Introduction

The functional aspects of cellular life are intrinsically tied to structure. Early observations of cellular ultrastructure by transmission electron microscopy (TEM) revolutionized the understanding of cell biology. Since then, sample preparation methods and TEM hardware have changed dramatically, opening up new routes to explore the complexity of biology with TEM. In particular, the vitrification of biological specimens has circumvented artifacts associated with sample preparation, allowing direct observations of untreated cells or purified biological complexes at high resolution. While the structures of purified protein complexes are now routinely determined by single particle analysis, where many images of copies of identical structures are averaged together [1], intact cells and heterogeneous or pleiomorphic biological structures are not amenable to such techniques. Electron tomography of vitrified samples (cryoET), wherein a series of images are taken of a single specimen at different angles relative to the electron beam and subsequently the three-dimensional architecture of the sample is computationally reconstructed, can be used to determine structures without relying on averaging or prior purification. An overview of cryoET can be found in Figure 1 and Ref. [2].

The study of protein complexes in their native environment at molecular resolution is challenging due to the complexity of the samples and the required preparation methods. In combination with techniques that allow the isolation of protein complexes on associated membranes [3], on the viral surface [4,5] or cellular extract [6,7], and the manipulation of cellular systems using molecular biology, cryoET is well placed to probe structure–function relationships inside cells. A strength of cryoET is the diversity of samples it can accommodate, from cells to reconstituted protein complexes. A prevalent class of cellular samples is unicellular organisms, ranging from common bacteria [8–14] (*Chlamydia* or *Bacillus*, for example) to single celled eukaryotes such as *Trypanosoma brucei* (the causative agent of African sleeping sickness) [15]. With cell thicknesses ranging from a few to several hundred nanometers, the periphery of these cells often can be imaged directly without need for prior specimen thinning (cf. the practical thickness limit for cryoET is ~1 μm at 300 keV accelerating voltage and using energy filtering). Heterogeneous membrane-enveloped viruses [5,16•,17] and bacteriophages [9,10] are likewise easily directly visualized by cryoET. Other eukaryotic cells can also be grown or placed on EM grids and imaged by cryoET [18–22], however their larger size and thickness (several micrometers) requires milling or sectioning in order to image details inside the bulk of the cell; although extreme regions such as the cell periphery still remain accessible without sectioning using judicious cell lines. An alternative to intact eukaryotic cells is work with a cell-derived system, such as isolated organelles [6,23,24••], extracellular vesicles displaying full-length over-expressed fusion proteins in their native membrane environment [3,25], or virus-like particles [26•]. Finally, components of the biological system of interest can be purified and reconstituted *in vitro* [21,27•]. Tomograms of samples prepared *in vitro* tend to be simpler to interpret, but lack the native cellular context.

Figure 1



An overview of cryoET. **(a)** Cells, viruses, or subcellular fractions can be used as specimens for cryoET. **(b)** (i) Specimens are grown or placed in buffer or culture medium on a mesh grid covered with a thin carbon layer punctuated by holes. (ii) Excess liquid is removed by blotting to leave just a thin layer before the grid is plunged into a bath of cryogen (iii) (typically liquid ethane) cooled to liquid nitrogen temperature (~77 K). Grids are then stored and/or directly imaged at liquid nitrogen temperature in an electron microscope. **(c)** Data acquisition and analysis in cryoET involves (i) tilting the specimen and taking a series of images collected at different viewing angles relative to the electron beam and (ii) subsequently, to computationally reconstruct from the tilt series images the object of interest in three dimensions.

The application of cryoET to probe diverse biological function necessitates the combination of technologies that increase signal-to-noise ratio and phase contrast in electron micrographs, especially at low electron doses [28,29]. In this review, we discuss recent technological advancements in cryoET which improve the determination of structural details of molecular processes *in situ* and expand upon the types of samples that can be visualized using cryoET. Recent themes in biological applications of cryoET, and uses of other methods complementing cryoET will also be addressed. Finally, we will address potential areas where cryoET has room for growth.

New technologies expand the applicability of cryoET to study macromolecular complexes in their native environment

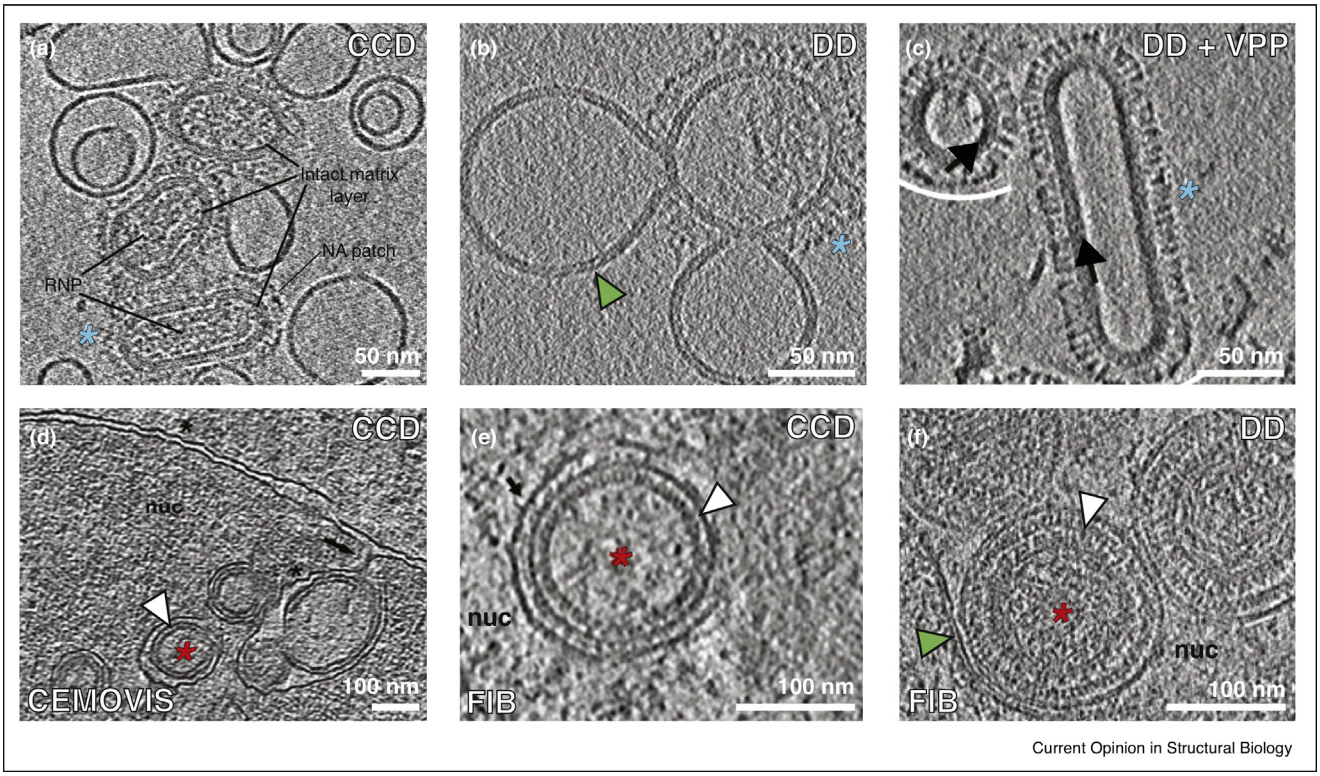
Like all modalities of cryoEM, cryoET has benefitted immensely from the development of direct electron detectors, which increase the signal-to-noise ratio (SNR) in images, and thus tomographic reconstructions [28]. However, three other recent methodological improvements have also changed the scope of what can be studied by cryoET.

Focused ion beam (FIB) milling [30–32] to thin vitreous samples has made the entirety of eukaryotic cells accessible to cryoET, beyond just the thinner peripheral areas previously imaged. Before the development of cryoFIB methods, vitreous sectioning (CEMOVIS) (see Figure 2d and Ref. [33]), a technically demanding technique, was the only approach for imaging thicker areas of vitrified cells. Although CEMOVIS is still in use for cryoET [15,34], many labs are switching to FIB milling [18,20,34]. Tomographic slices comparing different sample preparation and imaging methods are shown in Figure 2.

Another very recent technological development is the introduction of more robust phase plates for electrons [29,35]. Analogous to the phase plates used in light microscopy, Volta potential-based phase plates introduce an additional, relatively uniform, 90° phase shift to the scattered electron beam, increasing the phase contrast in the image. Traditionally, phase contrast in cryoEM has been generated by under focusing the microscope, resulting in uneven contrast across different spatial frequencies. As a new technology, the benefits and challenges of phase plate electron imaging are just beginning to be explored [19,26•], but the dramatically increased contrast could change standard practice in cryoET.

By contrast to single particle cryoEM, where the samples tend to be mostly homogeneous, cryoET is typically used to image very heterogeneous structures, with diverse morphologies. However, there may be repeating 'units' within that heterogeneity. Over the past few years, subvolume averaging has developed as a way to increase the SNR in reconstructions of such units. Unlike single particle cryoEM, where two-dimensional projection images are combined to produce a three-dimensional volume, subvolume averaging combines three-dimensional data. Subvolume averaging (Figure 3) has been successfully applied to tomograms of reconstituted or cell-derived systems [9,10,21,25,27•,36] or to tomograms

Figure 2



Examples of data from different sample preparation and imaging methods. **(a)** Tomographic slice of influenza virus fusing to liposomes, from [16], as recorded on a CCD detector, with the hemagglutinin fusion protein layer denoted by a cyan asterisk. **(b)** Slice through a tomogram of influenza virus-like particles fusing with liposomes taken from [26]. Data were recorded with a direct electron detector. **(c)** Slice through a tomogram of influenza virus-like particles also taken from [26]. The neuraminidase surface protein is indicated by the white lines, and the matrix layer by black arrows. Data were acquired using a direct electron detector and Volta phase plate. **(d)** and **(e)** Slices through tomograms showing vesicles (red asterisk) formed in the peri-nuclear space by the Herpes simplex virus nuclear egress complex (white arrow) [34]. Specimens were sectioned by CEMOVIS (d) and FIB milling (e), and images recorded on a CCD detector. The membranes in the sample prepared in (e) show clear signs of compression and distortion (black asterisk) due to the process of producing the vitreous section. Similarly, the order visible in the NEC complex in (e) and (f) is not fully preserved, and preclude subvolume averaging of the CEMOVIS data. **(f)** NEC complex in an infection setting (capsid cargo being present). Data was recorded under similar conditions but using a direct electron detector [43]. Note the increase in contrast and finer detail observable as we progress from a–c and d–f, especially in separation of the two leaflets of lipid bilayers (green arrow, b & f).

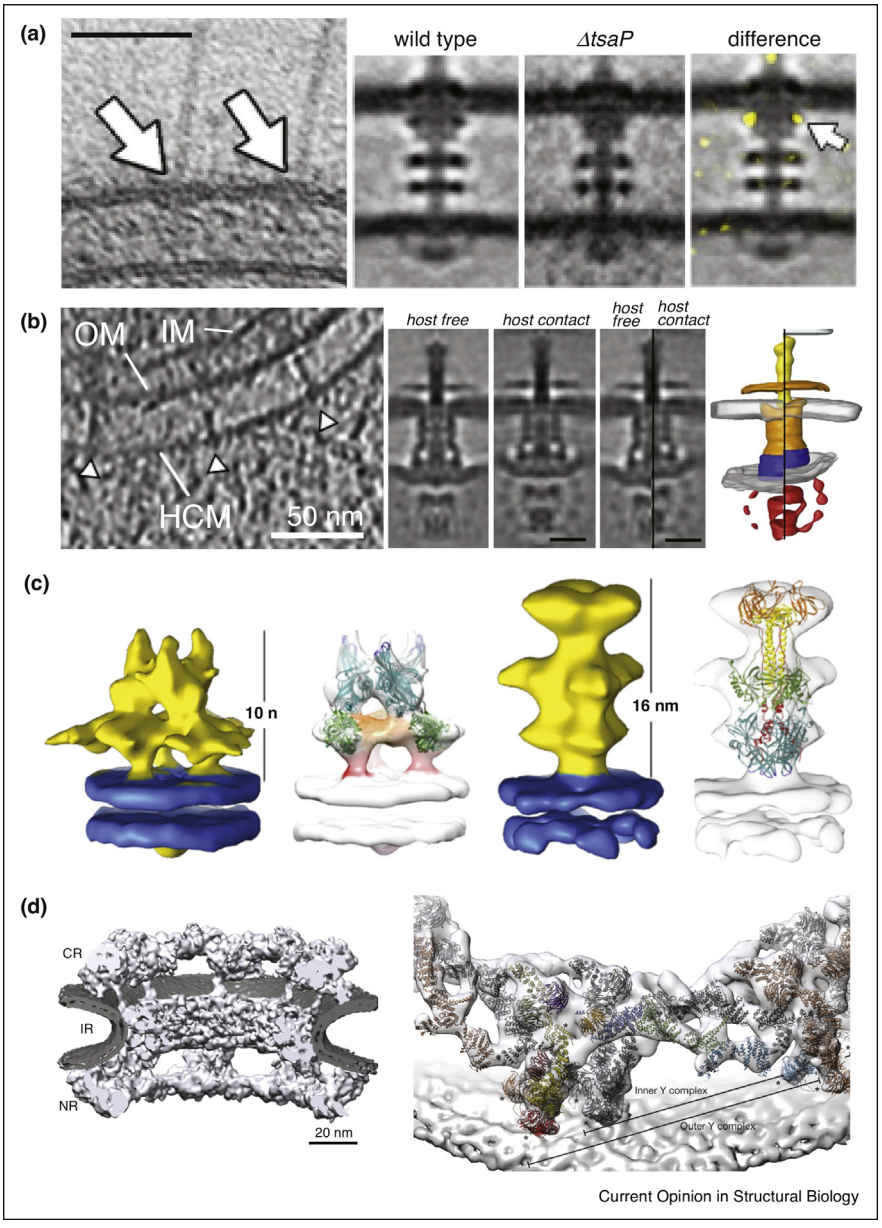
of cells or viruses [5,8,12••,17,19,20,34,37]. Very recently, the first near-atomic structure was determined by subvolume averaging of the capsid lattice from immature HIV [5], but most reconstructions are currently at resolutions of 1–2 nm. It remains to be seen whether phase plate imaging will improve the typical resolution of structures from subvolume averaging [38].

CryoET can be combined with multiple techniques in an integrative approach

One of the challenges of cryoET, especially for non-reconstituted samples, is the identification of specific molecules in tomograms. As such, cryoET is often paired with complementary methods to help untangle some of the cellular complexity (Figure 4). Genetic manipulation is often used in tractable organisms, especially bacteria. Knockouts and functional mutants can be screened for changes in cell morphology [11,12••], and fusion proteins can be added to localize specific subunits in complexes [12••]. Similar mutants can also be generated for some viruses [17]. A related technique for eukaryotic cells is heterologous protein expression. Comparison of different cell lines or cells with different levels of plasmid-driven protein expression can provide novel insights into function [34,36]. The full potential of new genome-editing techniques such as CRISPR/Cas-9 [39] in combination with cellular cryoET has yet to be explored.

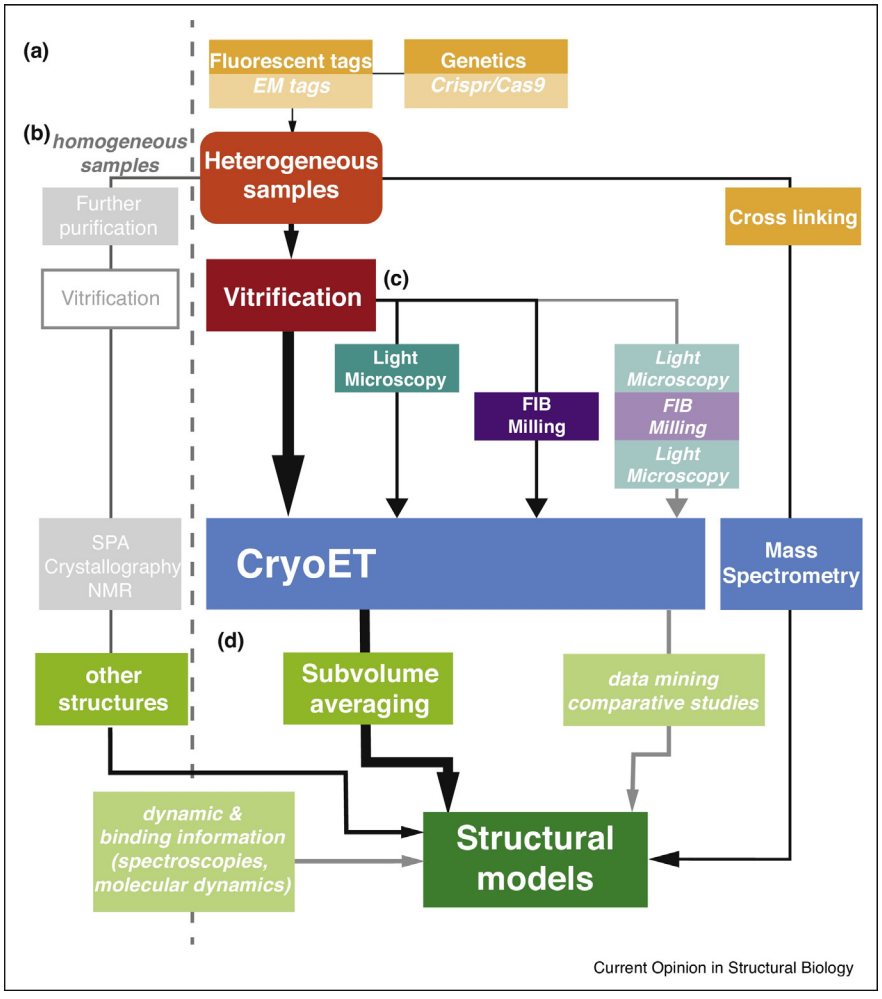
Another strategy for identification of proteins in tomograms is by comparison to structures derived from other techniques. X-ray crystallography-derived and single particle cryoEM-derived structures can be docked into cellular maps from tomography, to help localize and identify subunits [12••,23,24••]. In turn, tomography can be used to validate and provide context for structures determined *in vitro* [14]. CryoET is efficiently complemented by

Figure 3



Examples of structure determination and identification by subvolume averaging and complementary methods. **(a)** Localization of the Type IV pilus subunit TsaP by calculation of difference maps between subvolume averages of pili from wild type cells and ΔTsaP mutant cells (Figure adapted from [12]). **(b)** Slice through a tomogram of an elementary body of *Chlamydia*, inside a U2OS host cell (adapted from Ref. [8]). Shown is the contact between the secretion system of *Chlamydia* (white arrows) anchored in the outer (OM) and inner membranes (IM), and the host cell membrane (HCM). Subvolume averages of a host-free and host-bound state of the Pili are shown. **(c)** Atomic models of the domains from the post-fusion structure of the herpesvirus fusion protein gB (right) were fitted into another conformation (left) using integrated modeling methods (adapted from [25]). **(d)** Cross-linking and mass spectrometry were combined with subvolume averaging to guide fitting and identify positions of nuclear pore components within the nuclear pore complex (figure modified from [24]).

Figure 4



Workflow for cryoET, presently (bold, solid) and in the future (italics, transparent). **(a)** Currently, sample preparation steps use well-established tools such as fluorescent protein tags or gene knock-outs in tractable organisms, followed by vitrification. In the future, CRISPR/Cas9 technologies and new electron-dense tags will open up new avenues for cryoET. **(b)** From a heterogeneous sample (such as cells or subcellular fractions), traditional structural biology methods rely on additional purification to produce a homogeneous sample. Samples for cryoET can be vitrified without further processing. **(c)** CryoET can be preceded by correlative steps such as light microscopy to identify areas of interest, as well as thinning methods such as focused ion beam (FIB) milling to make thick specimens suitable for transmission electron microscopy. In the future, further automation will increase the ease and speed at which these experiments can be performed, generating larger data sets that can be used subsequently for improved subvolume averaging and other forms of data mining, comparing across many samples. **(d)** Structural models can be built by combining subvolume averages with structures and constraints from other methods. Cross-linking and mass spectrometry, as well as tagging methods, can be used to identify subunits in complexes and specific interfaces. In the future, further information about dynamics and binding will be incorporated into more sophisticated structural models enabled by more advanced computational approaches (cf. article by M. Topf and colleagues in this issue).

fluorescence microscopy: correlative light and electron microscopy (CLEM) can be used to identify regions of interest in cells, potentially also to guide FIB milling [30,40], before tomography (Figure 4c) [34]. CLEM is particularly useful when imaging rare events, as grids can be screened for areas of interest without having to take the time (and electron dose) to record a high magnification electron image. An emerging method for protein identification in tomograms is mass spectrometry. In complex samples, chemical cross-linking can be used to covalently attach neighboring molecules. Mass spectrometry can then be used to identify the proteins and their domains in physical proximity in structures of macromolecular complexes [24••,27•,36]. This approach is particularly useful when structures of individual subunits in a complex are unavailable, for disordered proteins, and for transient and instable structures where high resolutions maps by SPA are challenging to obtain. Examples of combining complementary methods for structural assignment with cryoET are shown in Figure 3.

CryoET allows the examination of a diverse array of biology

A particular strength of cryoET is in imaging membrane-related events, as the phosphate signal from the bilayer headgroups provides increased contrast. A recurrent theme in recent work is modulation of membranes during infections. Riedel *et al.* identified changes in the structure of the envelope protein of Murine Leukemia virus caused by interaction with host cell membranes [17]. Zeev-Ben-Mordehai *et al.* used native membrane vesicles to determine the structure of the pre-fusion form of the Herpes simplex virus 1 (HSV-1) fusion protein, gB [25]. Schur *et al.* used cryoET to understand assembly of the immature HIV capsid at high resolution, exploiting its high native symmetry [5]. Hu *et al.* [10] and Farley *et al.* [9] conducted similar experiments, but looked at changes in bacteriophage structure and host membranes upon binding to bacteria. Lastly, Stoeber *et al.* used cryoET to determine the structure of the protein coats surrounding cavaeolae [41].

Other recent work has focused on structures of endogenous membrane protein complexes. New insights into the architecture of the nuclear pore complex were provided by von Appen *et al.* [24••] and Kosinski *et al.* [36], while Mahamid *et al.* looked at the cellular ultrastructure in the nuclear periphery [18]. Dodonova *et al.* [27•] examined the formation of COPI vesicles in reconstituted systems, while Raschdorf *et al.* studied the formation of membrane-bound magnetosomes in bacteria [11]. Mühleip *et al.* [23] and Engel *et al.* [20] used cryoET to probe the protein arrangements underlying the ultrastructure of the mitochondria and the Golgi network, respectively, while Pfeffer *et al.* determined the structure of the translocon in the endoplasmic reticulum, which transports secreted proteins across the membrane [6].

Another area in which cryoET has provided biological insight is movement and motility. Tying back to the infection theme discussed earlier, Grange *et al.* examined virus trafficking along microtubules *in vitro* and in host cells [21]. Kawamoto *et al.* used cryoET to understand the unusual gliding movement of a pathogenic bacterium [13], while Chang *et al.* studied the structure and assembly of the widespread bacterial Type IV pilus system [12••]. Bharat *et al.* determined the structure of ParM filaments from bacteria, and imaged their activity in plasmid segregation *in situ* [14]. Similar to the pilus and ParM filaments, Höög *et al.* [14] examined the machinery responsible for flagellum formation in the unicellular parasite *Trypanosoma brucei*, while Jasnin *et al.* observed changes in actin structure in cells responding to an uneven growth surface [40].

One of the most exciting applications of cryoET is the potential for mechanistic understandings by capturing biological events in a process, as demonstrated effectively by four recent papers. Hagen *et al.* [34] used cryoET of milled and sectioned cells, combined with soft X-ray microscopy and correlated light microscopy, to visualize the assembly of a nuclear egress complex. In a subsequent paper, their subvolume average allowed docking of structures from X-ray crystallography with sufficient precision to propose a model for control of membrane curvature by the complex [42]. Chlanda *et al.* [26•] and Calder and Rosenthal [16•] used virus-like particles and intact viruses, respectively, to study membrane fusion driven by the influenza hemagglutinin protein. Chlanda *et al.* focused on lipid structures, describing the hemifusion diaphragm and a novel 'lipidic junction'. Calder and Rosenthal focused on the hemagglutinin protein itself, identifying its localization and structural rearrangements at different time points during the fusion process. Nans *et al.* [8] examined the interaction of a type III secretion system with host cell membranes during chlamydia infection. They identified conformational changes responsible for stabilizing interaction with the host membrane and leading to activation of secretion. Each of these papers, using either model or native systems, demonstrates the potential for cryoET to link structure with function (cf. Figures 2 and 3).

Conclusions

It is an exciting time for cryoET. The development of improved detectors and new specimen preparation techniques has opened up realms of biology which were previously intractable. CryoET combines exceptionally well with other methods, such as in cell NMR spectroscopy, mass spectroscopy, and fluorescence microscopy, to integrate structural and cellular biology. The potential of these hybrid methods is a move away from a 'reductionist' view of structural biology, to one where the biological context plays an important role in the structural analysis of a mechanism (Figure 4). New approaches to genetic modification such as CRISPR/Cas-9 [39] will further expand the power of cryoET. Advances in automation of data collection and processing, as well as further improvements in instrumentation such as detectors and sample thinning procedures, will increase the reliability and speed at which data can be collected. There is a need for suitable 'tags' for cellular cryoET, analogous to fluorescent proteins in light microscopy, ideally multimodal 'tags', to fully exploit the information present in tomograms. Further developments in the methodology will surely lead to a 'big data' era for cell biology — where studies across different systems can be compared to find common themes and differences in a longitudinal manner.

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