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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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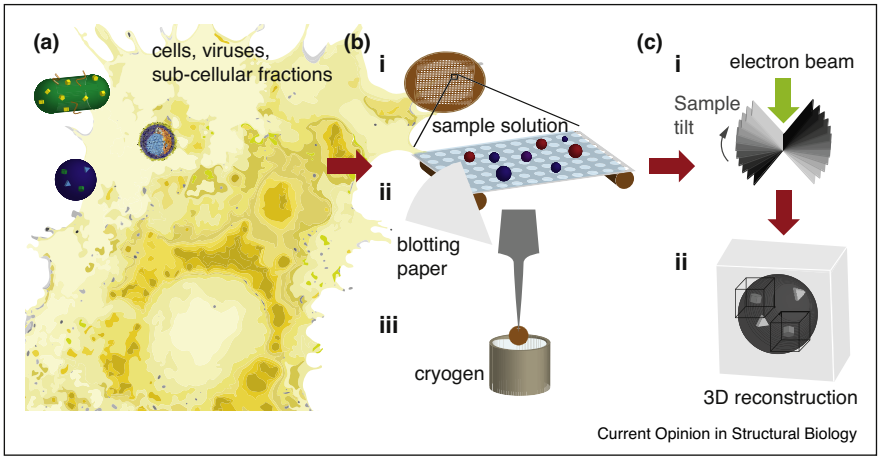
Introduction

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