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Commentary

Cryo-electron tomography: A long journey to the inner space of cells

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Cryogenic electron tomography (cryo-ET) is the application of tomographic principles of data acquisition and reconstruction to frozen-hydrated biological specimens. It combines a close-to-life preservation of cellular structures with the power of high-resolution three-dimensional imaging, which allows us to study the molecular architecture of cells, or their molecular sociology, in unprecedented detail.

Introduction

Cells are inhabited by myriads of different molecular species and the total population of proteins in any given cell can be somewhere between one or ten billion. Proteomics studies provide long lists of the molecular inventories of cells and organelles. For many molecular species, we know how they look in atomic detail. The divide and conquer approach of traditional structural biology has been tremendously successful. The richness of the protein database is testimony of this. However, proteins do not function alone; it is their collective behavior that underlies cellular functions. They must interact, stably or transiently, and act in concert to drive cellular physiology. David Goodsell's famous watercolors offer a vivid impression of the crowdedness and complexity of cellular environments but there is a need to visualize cellular landscapes in molecular detail as they actually are. Hitherto this has been largely an uncharted territory and exploring it is one of the grand challenges of cell biology (Asano et al., 2016). As we zoom in on protein complexes and reach high resolution, we can analyze the ensemble of conformational states that exists in a functional context. In tomograms we see not only what we look for, as is the case with imaging methods based on fluorescent labels, powerful as they are in revealing the positions of preselect targets, but also we get the whole picture. Cryo-ET provides holistic views of the inner space of cells and therefore has huge discovery potential. This is an opportunity as well as a challenge: an opportunity to observe the unexpected and a challenge to unravel and interpret the bewildering complexity of cellular tomograms.

Cells are organized in a number of distinct compartments to coordinate biochemical reactions. While membrane-bound compartments can be isolated, facilitating their structural analysis, membrane-less compartments or condensates formed by phase separation are more challenging to isolate and study. *In situ* studies by cryo-ET allow us to investigate the molecular populations inside these compartments without affecting their structural integrity.

The journey of cryo-ET to the inner space of cells has been a long and tedious one. Thirty years ago, it was demonstrated that by automation of the data acquisition, the cumulative electron dose needed for recording tilt series could be kept within tolerable limits for the investigation of radiation-sensitive materials such as ice-embedded biological samples (Dierksen et al., 1993). Initially, only small objects such as virus particles or small prokaryotic cells were accessible for cryo-ET (Grünewald et al., 2003). Eukaryotic cells grown on or deposited onto electron microscopy (EM) grids were too thick to be electron transparent in their entirety; only thin peripheral regions or appendages could be investigated (Medalia et al., 2002). This changed with the advent of focused ion beam (FIB) milling, opening virtually "windows into cells" (Rigort et al., 2012). Still, the workflow beginning with vitrification by rapid freezing to thinning of the samples by FIB milling followed by transfer to the electron microscope for the acquisition of tilt series to the reconstruction of the 3D volumes and their interpretation is long and often cumbersome. As a consequence, throughput is limited with current implementations of the method and for the realization of the full potential of the method advances on several frontiers are needed (Bäuerlein and Baumeister, 2021).

In the following, some of the limitations of the method will be discussed along with prospects to overcome them.

The cryo-ET workflow

The first step in the workflow is cryopreservation by vitrification. For small objects, such as single cells grown on or deposited on EM grids, this can be accomplished by plunge freezing. With larger objects, such as tissues or multicellular organisms, high-pressure freezing is needed to achieve vitrification. This results in relatively thick blocks of ice complicating the targeting of volumes of interest and the preparation of lamellae including them. The localization of features or events of interest is achieved by cryo-correlative microscopy (cryo-CLEM). This approach takes advantage of the strength of light microscopy (LM) in identifying preselect fluorescently labeled structures and guides the cryo-FIB lamella preparation. Currently, the two steps, namely the localization by cryo-fluorescence microscopy and FIB milling, are executed in separate instruments but would obviously benefit from integration to facilitate the alignment of the coordinate systems of the LM and FIB and eliminate the risk of ice contamination during transfer between the instruments.

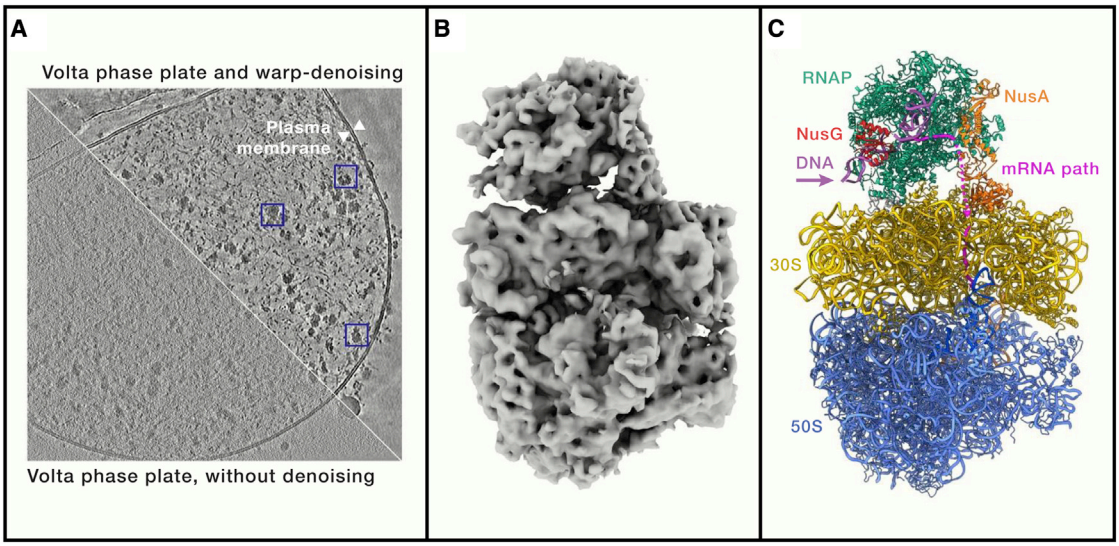

Figure 1. Visualizing transcription-translation coupling inside *Mycoplasma pneumoniae* cells

(A) Tomographic slices of a typical *M. pneumoniae* M129 cell imaged with the Volta phase plate (VPP). The noise2noise network implemented in Warp/M (Tegunov et al., 2021) was applied to boost contrast. Obvious features such as the plasma membrane, ribosomes (blue boxes), etc. are highlighted.

(B) The supramolecular assembly consists of ribosomes and RNA polymerase, determined by classifying the ribosome sub-tomograms extracted *in silico* from the cellular tomogram.

(C) The derived integrative model of an actively transcribing and translating expressome demonstrated a novel transcription-translation coupling mechanism mediated by NusA. The genetic information flow from DNA to mRNA to the nascent peptide is directly visualized in the expressome complex inside bacterial cells. By courtesy of Liang Xue and Julia Mahamid (see O'Reilly et al., 2020).

The preparation of lamellae by cryo FIB milling has become the standard procedure for the thinning of cellular samples. However, it is a throughput-limiting factor. Even though it is increasingly accelerated by automation, it remains a bottleneck. Future instruments using a plasma source instead of a gallium beam are expected to speed up lamella preparation allowing also to produce lamellae from volumes deep inside tissues.

Another factor limiting throughput is the speed of data acquisition in the transmission electron microscope (TEM). The recording of tilt series, comprising typically ~100 projection images, takes about 20–40 min but can be speeded up substantially by elaborating fast-tilt acquisition schemes. With future improved specimen stages and faster cameras, it should be possible to take complete datasets in less than a minute (Chreifi et al., 2019).

In cryo-ET, the signal-to-noise (SNR) of the images is necessarily low because of the need to minimize exposure of the specimens to the electron beam. The options for improving the SNR by averaging, which is common practice in cryo-EM single-particle analysis (SPA), are much more limited in cryo-ET because of the omnipresent stochasticity of cellular structures. Therefore, it is important to maximize phase contrast, e.g. by using phase plates similar to the Zernicke phase plate used in LM. Usage of the Volta phase plate has proven useful for the detection of small and variable structures in crowded cellular environments. It has allowed researchers to visualize previously undetectable structures in tomograms (Pöge et al., 2021). However, it is not a perfect solution and there is hope that the laser phase plate, which is currently under development, will become a near-perfect solution, allowing scientists to push the boundaries of cryo-ET further (Schwartz et al., 2019).

Data mining and interpretation

Some of the imperfections of tomographic datasets, such as resolution-limiting beam-induced motions, can be removed after acquisition by software tools. Attempts to mitigate the effects of missing data due to the limited tilt range are still in the beginning stages but could benefit from machine learning approaches. The same is true for denoising procedures suppressing noise while maintaining the signal. Denoising facilitates the segmentation of the tomograms and particle picking for subsequent classification and averaging. Macromolecules can be localized and identified by template matching using an *a priori* known reference structure either derived from experimentally determined structures or predicted by methods such as Alpha Fold. Alternative template-free approaches for detecting and picking densities based on machine learning methods hold promise for a more comprehensive and unbiased interpretation of tomograms (Moebel et al., 2021).

Subtomogram averaging of particles detected by either method yields structures with improved signal-to-noise ratio and resolution. There are two major limitations: the size of the particles and their abundance. With current implementations of cryo-ET, the lower size limit is somewhere between 200 and 400 kDa, depending on the shape of the molecules and their environment. Unlike in SPA, where the number of copies for averaging is essentially unlimited, in cryo-ET the copy numbers are given by nature and can range from a handful or less to a

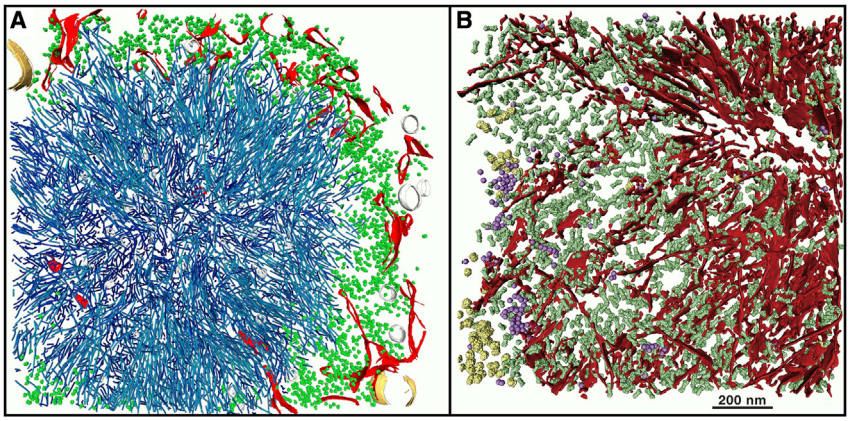

Figure 2. *In situ* structures by cryo-ET of two kinds of neurotoxic aggregates
Poly Q inclusions the hallmark of Huntington disease (A) and Poly-GA aggregates, the hallmark of amyotrophic lateral sclerosis (B). For references see Bäuerlein et al. (2017) and Guo et al. (2018). The toxic mechanisms suggested by cryo-ET are obviously quite different.
(A) Interactions between the Poly Q fibrils (blue) and the ER (red) lead to the disruption of the latter by changing local membrane curvature and leading to their disruption releasing ribosomes (green).
(B) Poly-GA aggregates consist of polymorphic ribbons (red) massively accumulating and stalling 26S proteasomes (green) thereby compromising cellular protein quality control. Ribosomes (yellow) and the chaperonin TRiC (purple) are excluded from the aggregate.

few thousands in a single tomogram. With large and high abundance complexes, such as ribosomes or expressosomes, near atomic resolution has been attained (O'Reilly et al., 2020; Figure 1). In less favorable scenarios, resolution is lower. However, when the identity of a structure obtained by subtomogram averaging is known, hybrid or integrative approaches allow for upgrading them to pseudoatomic structures by the integration of high-resolution experimental structures or predicted structures of components. Moreover, not in all cases is it necessary to attain near-atomic resolution. What is important is to attain a resolution relevant to the problem and often enough is contextual information more important than resolution. For example, for illuminating the mechanisms by which neurotoxic aggregates damage or perturb their cellular environments, there is no need to reach resolution in the subnanometer range (Figure 2; Bäuerlein et al., 2017; Guo et al., 2018). It would be desirable to develop formal quantitative measures or criteria for the information density and content of tomography data.

The identification and annotation of densities detected in tomograms is a weakness of cryo-ET. Attempts to create genetically encoded labels suitable for cryo-EM have hitherto proven unsatisfactory for a variety of reasons. A possible solution to this problem comes from cryo-CLEM. Taking advantage of the strength of super-resolution LM in identifying and localizing molecular species, one could map a single molecule fluorescent signal onto a tomogram to determine or at least suggest the identity of an observed density.

Since small molecules may escape detection and identification, one can take advantage of orthogonal data to complement the molecular maps provided by cryo-ET. Larger complexes could serve as anchor points and their surroundings be modeled based on proximity information derived from *in vivo* crosslinking and mass spectrometry (Lenz et al., 2021).

Concluding remarks

From humble beginnings three decades ago, cryo-ET has evolved steadily into a powerful tool for structural cell biology or visual proteomics. It has unique potential to generate molecular maps of cellular landscapes and in favorable cases it has reached near-atomic resolution. Ongoing developments in technology and methodology hold promise to push the boundaries further toward completeness of the maps and resolution of *in situ* structures. That is where many see the future of structural biology.

DECLARATION OF INTERESTS

W.B. is on the Life Science Advisory Board of Thermo Fisher Scientific.

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