Biomolecular Electron Microscopy: From Molecules to Systems

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Today's biomolecular electron microscopy uses essentially three different approaches. (i) electron crystallography, (ii) single particle analysis and (iii) electron tomography.

Ideally, these imaging modalities are applied to frozen-hydrated samples to ensure an optimum preservation of the structures under scrutiny. Given the radiation sensitivity of biological materials embedded in ice, it is indispensable to collect data under low-dose conditions.

Electron crystallography requires the existence of two-dimensional crystals. It has been particularly successful with membrane proteins; their properties favor the formation of 'membrane crystals'. In principle, electron crystallography is a high-resolution technique and it has indeed been demonstrated in a number of cases – some of the high-profile structures – that near-atomic resolution can be attained. Nevertheless, it is notoriously difficult to collect high-resolution data at higher tilt angles for reasons which are not yet fully understood. The difficulties encountered in obtaining well-ordered two-dimensional crystals and the low yield of high-resolution images limit throughput in electron crystallography and often put it at a disadvantage in competition with other methods, in particular x-ray crystallography.

Single-particle analysis is arguably a misleading term since it involves the averaging over large numbers of individual particles. It is based upon the assumption that the particles being studied occur in random orientations when embedded in a thin layer of ice. Following the classification of the different views, class averages are generated and their relative orientation is determined; they are then assigned a place in a virtual tilt experiment, and, finally, the three-dimensional reconstruction is performed. Single-particle analysis is particularly suited for structural studies of large macromolecular complexes. The amounts of material needed for the analysis is minute and some degree of heterogeneity (impurities, conformational variability) is tolerable since image classification can be used for further 'purification *in silico*'. In principle, single particle analysis can attain high-resolution but, in practice, this often remains an elusive goal. However, since medium resolution (~1 nm) structures can be obtained relatively easily, it often provides an excellent basis for hybrid approaches in which high-resolution structures of components (subunits, domains) are integrated into the medium resolution structures of the holocomplexes. In many cases such hybrid approaches can provide most valuable structures with pseudoatomic resolution [1].

Unlike electron crystallography and single particle analysis, both requiring deterministic and, hence, repetitive structures, electron tomography can be applied to structures which are subject to stochastic variations and therefore non-repetitive. Most supramolecuar structures inside organelles and cells fall into this category. In order to obtain three-dimensional structures of objects with unique topologies it is necessary to obtain different views by physical tilting. The challenge is to obtain large numbers of projection images covering as wide a tilt range as possible and, at the same time, to minimize the cumulative electron dose. To achieve this, the allowable electron dose (i.e. the dose not

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causing visible damage) is fractionated over the total number of projections with the aid of automated acquisition methods that, at the same time, compensate for small deviations from perfect eucentricity of the tilting device. Elaborate microscope control protocols compensate for the resulting image shifts and changes in focus [2]. Cryoelectron tomography provides medium resolution (2-4 nm) three-dimensional images of a wide range of biological structures from isolated macromolecular assemblies to organelles and cells [3]. It allows the visualization of molecular machines in their functional environment and the mapping of entire molecular landscapes, i.e. the generation of molecular atlases from which interaction networks can be deduced [4,5].

In this presentation two large proteolytic complexes will be discussed in some detail which exemplify the potential of single particle analysis and the usefulness of hybrid approaches (Fig. 1).

The 26S proteasome plays a key role in intracellular protein quality control. It is the most downstream element of the ubiquitin-proteasome pathway of protein degradation. Proteins carrying multiubiquitin chains as a death signal are selected by the 26S complex and degraded in an ATP-dependent manner [6,7]. The 26S holocomplex comprises 34 different constitutive subunits and has a molecular mass of 2.5 MDa. It is a highly dynamic and a very labile structure. As a consequence, very large data sets must be recorded, preferably by automated methods, which allow to capture subtypes of the 26S structure [8]. For some modules of the complex, such as the 20S core complex or the ATPase ring atomic or pseudoatomic resolution structures exist, which can be fitted into the EM envelope of the holocomplex. There is also a substantial body of proteomics data specifying binary interactions between subunits which can be used to further interpret the EM map. Taken together, these data allow to put forward a detailed structural model of the 26S complex providing insights into the mechanism of action of this intriguing molecular machine [9].

The second example illustrating the power of single particle EM and of hybrid approaches is tripeptidylpeptidase (TPP)II. It probably has the distinction of being the largest enzyme complex studied in atomic detail thus far. This giant protease has a molecular mass of 6 MDa. It augments the function of the proteasome and can under certain circumstances substitute for it. The structure of the spindle-shape complex, comprising two twisted strands of 10 dimers of the 150 kDA subunit each, has been determined by single particle analysis to a resolution of 1.4 nm. The structure of the 300 kDa dimer has been determined by x-ray crystallography. The x-ray map has then been docked into the EM map to provide a pseudoatomic model. The salient feature of the hybrid structure is an elaborate network of intramolecular channels and cavities harboring the active sites and controlling the access of substrates [10,11,12].

To illustrate the opportunities provided by electron tomography recent work on the nuclear pore complex and on polyribosomes will be discussed (Fig. 2).

Nuclear pore complexes reside in the nuclear envelope of eukaryotic cells where they mediate the nucleocytoplasmic traffic of macromolecules. The structural analysis of the nuclear pore complex is a formidable challenge given its size, its location in a membraneous environment and its dynamic nature. Electron tomography has allowed to study nuclear pore complexes in their functional environment, i.e. in intact nuclei. This avoids the risk of structural changes associated with their isolation from nuclear envelopes and it maintains their functionality, here the ability to translocate cargo. From tomograms of intact nuclei subtomograms including individual nuclear pore complexes have been selected and excised for alignment and subsequent averaging. An image processing strategy compensating for distortions of the eight-fold symmetry of the individual nuclear pore complexes resulted in a significant improvement in resolution. Furthermore, the superposition of a large number of tomograms taken in the presence of cargo, which was rendered visible by gold nanoparticles,

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has yielded a map outlining the trajectories of import cargo. This demonstrates that electron tomography, in spite of being a static method, can provide snapshots of dynamic processes [13,14].

The ribosome has been the subject of numerous studies by x-ray crystallography and cryoelectron microscopy in recent years and this has greatly advanced our understanding of the translation machinery. In contrast, very little is known about the spatial arrangements of individual ribosomes during active protein synthesis. Linear arrays of polyribosomes have been observed by traditional EM studies, but the three-dimensional organization of these flexible arrays remained elusive. Electron tomography has allowed to perform detailed structural studies of ribosomes, both in cell lysates and in intact cells. Cell lysates allow the unambiguous identification of polysomes and manipulation of the system. *In situ* studies corroborate that the topologies observed *in vitro* represent the *in vivo* configurations. Analysis of characteristic examples of polysomes reveals a staggered or pseudohelical arrangement of ribosomes along the mRNA trace, with the transcript sequestered on the inside, the tRNA entrance sites being exposed and the polypeptide exit sites facing the cytosol. This configuration has important implications for protein synthesis and folding in cellular environments [15].

Cryoelectron tomograms of organelles or cells contain an imposing amount of information; essentially they are three-dimensional representations of the entire proteome of cells. However, mining this information is not a trivial task because the signal-to-noise ratio of the tomograms is low and individual macromolecules are hard to recognize in an environment that is so crowded that they literally touch each other. There are basically two alternative (but not mutually exclusive) approaches for mapping macromolecules in cellular environments: Specific labeling with electron-dense markers and computational strategies based on innate structural signatures and pattern recognition. Strategies based on labeling cannot detect more than a tiny fraction of the proteome simultaneously in any given cell. Computational methods, however, enable one to interrogate and interpret tomograms in a comprehensive manner. Methods based on pattern recognition are more demanding in terms of resolution and they require *a priori* knowledge of the proteomic inventory as well as of the structures under scrutiny [16,17].

Further advances in technology and in methodology are needed to achieve the ambitious goal of a comprehensive molecular mapping of cellular landscapes. The current resolution of typically 4 nm achievable with cellular structures allows only large macromolecular structures to be identified and mapped with an acceptable fidelity. But it is a realistic prospect that with improved instrumentation resolutions around 2 nm are within reach. Hybrid approaches such as the combination of light microscopy and electron microscopy as well as of microdissection and mass spectrometry will enable us to precisely target cellular structures and to reveal the macromolecular organization underlying cellular functions [18].

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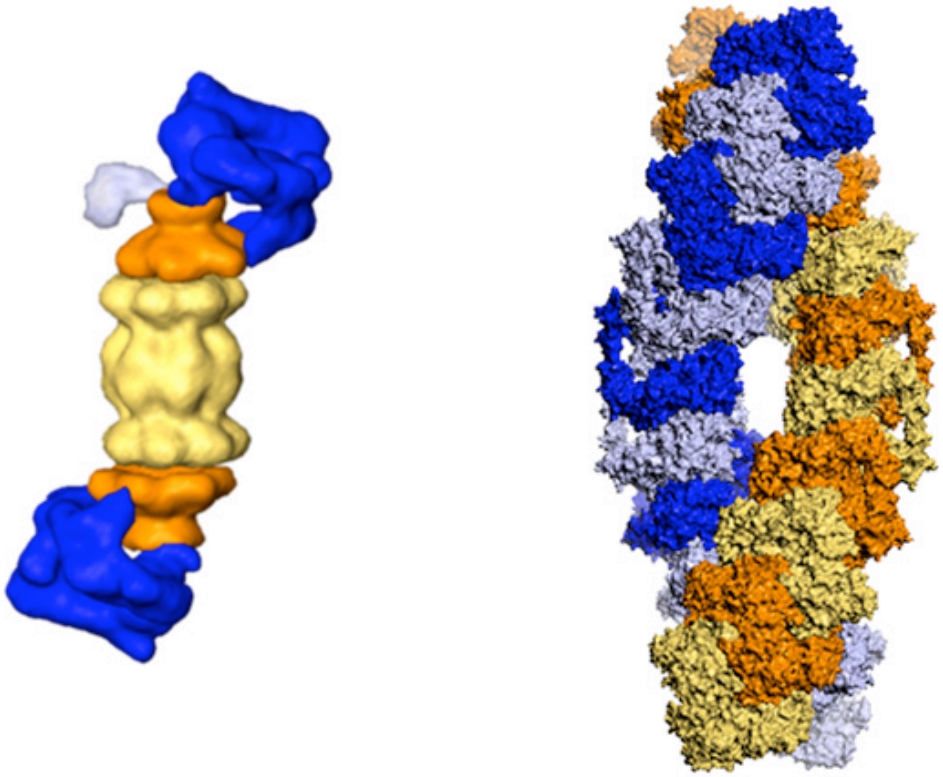

Figure 1. Structures of the 26S proteasome (left) and tripeptidylpeptidase (TPP) II (right) as determined by single-particle analysis (26S) and a hybrid single-particle EM – x-ray crystallography approach (TPPII).

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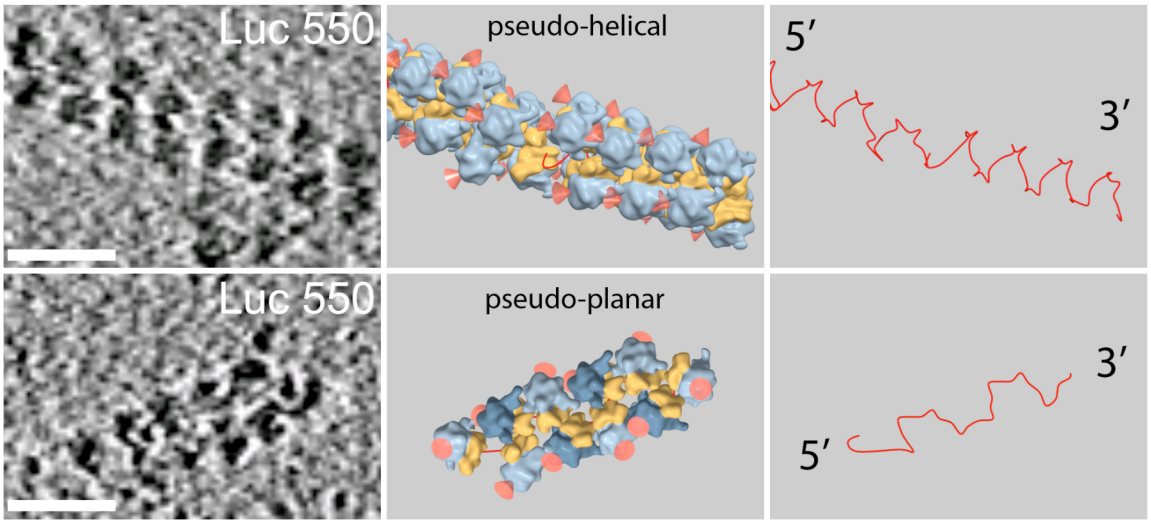

Figure 2. The native organization of bacterial polysomes as observed in an *in vitro* translation system. Two basic configurations have been observed: pseudohelical and pseudoplanar. Left column: Slice through tomograms. Center column: Interpretation based upon template matching. Right column: Deduced putative path of mRNA (from ref. 16).

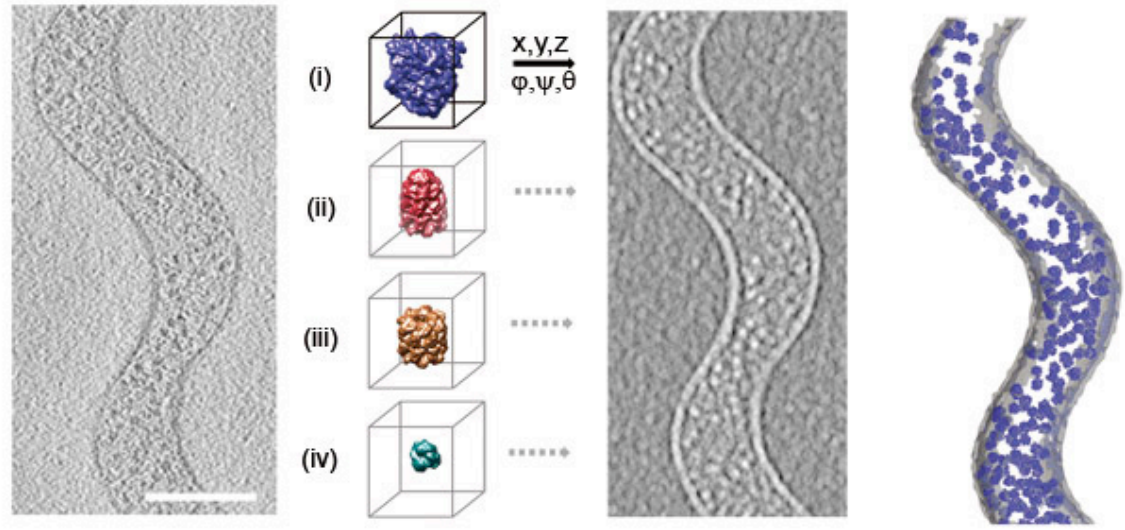

Figure 3. Interpretation of an electron tomogram of the bacterium *Spiroplasma melliferum* by pattern recognition. The tomogram (left) is cross-correlated with a library of template structures (center); the resulting cross-correlation peaks represent the positions and orientations of the molecules under scrutiny. This allows to establish a molecular map of the cell (from ref. 18).

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