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Review

Imaging of organelles by electron microscopy reveals protein–protein interactions in mitochondria and chloroplasts

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ABSTRACT

Ongoing progress in electron microscopy (EM) offers now an opening to visualize cells at the nanoscale by cryo-electron tomography (ET). Large protein complexes can be resolved at near-atomic resolution by single particle averaging. Some examples from mitochondria and chloroplasts illustrate the possibilities with an emphasis on the membrane organization. Cryo-ET performed on non-chemically fixed, unstained, ice-embedded material can visualize specific large membrane protein complexes. In combination with averaging methods, 3D structures were calculated of mitochondrial ATP synthase at 6 nm resolution and of chloroplast photosystem II at 3.5 nm.

1. Introduction

A first step in comprehension of metabolic processes is an understanding of protein–protein interactions within organelles or even complete cells. This goes beyond the level of the visualization of a cell ultrastructure with its nucleus, mitochondria, endoplasmatic reticulum and other organelles. By fluorescence microscopy many conclusions can be drawn about subcellular localizations, and nowadays there is a whole toolbox of fluorescent proteins assessing protein location and function [1]. But to understand protein–protein interactions we need a toolbox beyond the micrometer scale of light microscopy. Ongoing progress in electron microscopy (EM) offers now an opening to visualize cells at the nanoscale. The first attempts to understand processes, such as cargo transport through nuclear pore complexes in intact nuclei, can be solved with samples that did not undergo extensive chemical treatment, fixation and staining [2], in marked contrast to the ones on which the first concepts of subcellular ultrastructures were developed in the 1950s of the last century. In this contribution, we will discuss two techniques from the electron microscopy toolbox, electron tomography and single particle averaging, which are useful utensils in structural biology. We further give examples of these techniques, dealing with organelles with a highly complex system of inner membranes, in which protein–protein interactions are crucial for efficient energy conversion.

2. Techniques for study of subcellular structures and large biomacromolecules

Electrons can be accelerated to produce beams with a much shorter wavelength than of visible light. This allows EM to be performed at atomic resolution. Contrast in EM arises from elastic scattering of electrons. For weakly scattering objects, such as biological material, phase contrast is the most important part of contrast [3]. The signal is proportional to the electron dose, but unfortunately the electron beam also damages biological molecules because inelastically scattered electrons deposit their energy in the specimen. Because radiation damage should be kept at a minimum this results in noisy images, especially for biological objects. Therefore, image analysis techniques have been developed to improve the signal recorded by averaging over many projections of the same molecule. Single particle averaging is nowadays the most popular way of improving the signal-to-noise ratio.

Another point is the fact that EM produces 2D projections of the imaged 3D objects. The superposition of features along the electron beam direction results in images that can be ambiguous and deceptive. To retrieve 3D information of subcellular structures, tilting of specimens in the electron microscope is necessary. To image 3D objects electron tomography (ET) has been developed. In ET, the

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third dimension is retrieved by combing dozens of images recorded under different tilt angles.

2.1. Single particle averaging

Single particle averaging has become popular because it can deal with randomly oriented single molecules, which is a great advantage over methods that need protein crystals. By adding hundreds or, if possible, hundreds of thousands of projections the signal improves substantially and trustworthy electron density maps are obtained. The method of single particle analysis consists of a few dedicated steps (see [4–7] for reviews on single particle EM). Because single particles have random orientations on the support film, averaging is possible after an alignment step. The alignments bring projections in equivalent positions by computing rotational and translational shifts. After alignment, particles can be summed and by increasing the number of summed projections the noise is gradually fading away. Just summing of projections, however, is meaningless because they usually arise from particles in different orientations toward the plane. In order to deal with this, data sets have to be treated with multivariate statistical analysis together with automated classification [4,5] to separate different angular projections before they can be averaged. The fact that single particles are randomly oriented in a thin layer of amorphous ice has also led to the development of 3D reconstruction methods that take advantage of this [8].

Close to atomic resolution has been achieved by single particle averaging for some biomolecules bearing high symmetry, which enables interpretation of electron density maps at the level of amino acids [9]. For a stable, non-symmetric particle like the ribosome fitting of the polypeptide chain is yet possible, but without highest accuracy [10]. Many large protein complexes are, however, somewhat flexible. This severely limits the possibilities to obtain subnanometer resolution [7], as obtained for the ribosome. On the other hand, even lower resolution data of large multi-subunit complexes can be very useful, in particular if they can be combined with high-resolution structures of components. Fitting of X-ray data into EM data is useful if the EM data are better than 15 Å, because fitting yields hybrid structures which are about 5 times better than the resolution of the EM structure [11]. This means that it is already possible to look at helix–helix interactions with EM 2D maps and 3D volumes slightly better than 15 Å.

2.2. Electron tomography

Electron tomography (ET) can provide three-dimensional information on any object with a thickness up to about 0.5 μm and is very suitable to study biological structures with nanometer resolution (see [12–14] for recent reviews). It can be applied on quickly frozen samples and imaging of unstained material in an amorphous layer of ice makes cryo-ET remarkably versatile, allowing the exploration of a large range of biological specimens, both in an isolated state and in their cellular context. By itself, ET is not an averaging technique, because it only reconstructs a 3D volume, the tomogram, by a combination of dozens of projections taken at different tilt angles. However, by extracting sub-volumes corresponding to specific cellular components such as membrane proteins that are present in multiple copies, 3D averaging can be used to obtain density maps of selected macromolecular complexes at progressively higher resolution [12]. This brings ET in the realm of single particle averaging and many of the tools used in the latter technique can be applied in ET as well [6]. Sorting of molecules, for instance, demonstrated the ability of image classification to successfully separate the different conformations of the HIV-1 envelope glycoprotein spikes [15]. The newly emerging methods that combine cryo-ET with 3D image classification and averaging yield 3D images of molecular machines in a native environment, in a variety of biochemically well-characterized states, unconstrained by intermolecular contacts characteristic for a crystal [16].

In the next two sections we provide some examples from mitochondria and chloroplasts that illustrate the possibilities of single particle averaging and electron tomography, with an emphasis on the membrane organization.

3. Mitochondrial supercomplexes

The organization of cristae membranes within mitochondria is a very relevant topic because it now appears that the complexes involved in the oxidative phosphorylation system (OXPHOS) influence the morphology of these membranes. Moreover, it has been found that the OXPHOS complexes form supramolecular assemblies termed respiratory supercomplexes or respirasomes [17]. In recent years, the structure of the most relevant supercomplexes has been determined by single particle analysis at medium resolution, after membrane solubilization and protein purification. The most detailed data are from a yeast supercomplex formed by dimeric complex III and two monomeric copies of complex IV [18]. Fitting X-ray data with EM maps resulted in a pseudo-atomic model, describing positions of subunits involved in electron transfer between complexes III and IV. After the discovery of supercomplexes it was questioned if these structures could be detergent artifacts. Single particle averaging indicates this is unlikely because they show specific interactions. If these supercomplex structures would be artifacts, single particle analysis should have failed to reveal fine details. Nevertheless, the possible influence of detergent has to be faced. One aspect is that upon solubilization transient binding subunits are easily lost. An analysis of ~80 000 particles of the III_2–IV_2 supercomplex of yeast showed that only a couple of dozen cytochrome *c* proteins remained attached to this supercomplex. There are also other components that could transiently bind to the OXPHOS supercomplexes. For instance, the 30 kDa ADP/ATP carrier loosely binds to the III_2–IV_2 supercomplex, but its association is sensitive to detergent levels [19], and has not yet been detected in EM projection maps.

The possible loss of supercomplex components and the difficulties to study protein–protein interaction after solubilization prompted us to focus more on ET. Previously, the cristae structure was studied by conventional EM methods, including chemical fixation and sectioning. This led to the standard baffle model for cristae structure that later was clearly shown to be inaccurate [20]. Depending on source and conformational state, cristae can vary from simple tubular structures to more complex lamellar structures merging with the inner boundary membrane through tubular structures 28 nm in diameter [20]. The structural information provided by cryo-ET can offer a lot of insight how supercomplexes are distributed over the cristae membranes. Because tilting of samples in a microscope is restricted to about 60–70°, it means that a substantial part of the 3D space is not sampled. This missing information is reduced if a second tilt series is recorded after rotating the sample by 90°. Thus dual-axis ET provides a way to see subcellular structures at higher resolution [21]. We applied dual-axis cryo-ET to intact, close-to spherical mitochondria from the alga *Polytomella* [22]. A slice through the center of an electron tomogram is presented in Fig. 1A. The difference in thickness between the outer membrane and inner membrane is clearly visible. In this particular mitochondrion, cristae are abundant; the slice suggests that they are separated, but surface rendering of the membranes indicates that they are interconnected. Surface rendering is also a tool to visualize the folding of the inner membrane (coloured brown and yellow, Fig. 1B) and to get an impression of the protein