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STRUCTURE OF MACROMOLECULAR COMPOUNDS

Cryo-Electron Tomography Studies of Cell Systems

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Abstract—Cryo-electron tomography is a powerful tool for determining the three-dimensional structures of macromolecular complexes in their natural environment. The minimization of external impacts on the investigated objects, possibility of conducting *in vitro* and *in cellulo* experiments under close-to-native conditions, and high spatial resolution of the obtained three-dimensional reconstructions make cryo-electron tomography one of the most promising methods for studying a large class of objects in the fields of structural biology and visual proteomics. The main aspects of cryo-electron tomography are discussed as applied to studies of cell systems.

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INTRODUCTION

The development of methods of cryo-electron microscopy (cryo-EM), especially pronounced in the last years [1], has significantly increased the number of macromolecular structures deciphered with a near-atomic resolution. The best known cryo-EM method, single particle analysis (SPA), has become popular as a powerful tool for studying single proteins and protein complexes in the near-native state. The name of this method reflects its main scenario, i.e., study of multiple copies of single protein macromolecules with a mass between 100 kDa–100 mDa [2] in different orientations, averaging the data (tens of thousands of images of the object projections), and reconstructing a high-resolution three-dimensional structure on their basis.

Simultaneously, the method of cryo-electron tomography (cryo-ET) has been significantly developed. This technique provides a unique opportunity for direct visualization of molecular structures in their natural functional environment [3]. This method is mainly aimed at studying macromolecular complexes with variable morphology, as well as viruses, bacteriophages, and cells.

Cryo-ET is applied to objects for which structural information cannot be obtained by other methods for some reasons, for example, in view of their non-crystallizability, large size, and difficulties with extracting and cleaning samples. The use of cryo-ET, in turn, makes it possible to study the complexes that are difficult to reproduce and extract directly in cells [4], as well as their conformations, mutual orientations, and interactions. As a rule, less than 1000 particles of an object of interest are used to process data and obtain high-resolution structures using the cryo-ET technique. Thus, at present, cryo-ET fills the gap in physical dimensions of investigated objects and the spatial resolution of the data between super-resolution optical microscopy and atomic-resolution methods for determining the structure of biological macromolecules, including nuclear magnetic resonance, SPA, and X-ray diffraction analysis.

The use of a complex of methods, e.g., vitrification, cryo-fluorescence microscopy, cryo-scanning electron and ion microscopy, and cryo-ET, makes it possible to obtain three-dimensional cell structures, including their internal filling, and investigate intercellular and cell–matrix contacts in the native state on different spatial scales. In the coming years cryo-ET can be expected to become one of the main structural methods of cell biology. *In situ* cryo-ET can combine most of existing approaches to study the cell microstructure at a high spatial resolution for macromolecules in an intact cellular environment. In combination with modern methods for sample preparation using vitrification procedures and a focused ion beam (cryo-FIB) to obtain thin lamellae of frozen hydrated samples, as well as applying the latest data processing software, the use of cryo-ET will undoubtedly lead to discovery of new macromolecular and supramolecular structures.

TECHNICAL ASPECTS OF CRYO-ELECTRON TOMOGRAPHY

The main idea of the cryo-ET technique is the possibility of reconstructing a three-dimensional struc-

ture of an object from a sufficiently large number of projection images of object, obtained in a wide range of tilt angles [5]. This technique is mainly used to examine essentially heterogeneous objects with a diverse morphology [6]. The use of sub-tomogram averaging for repeating structural elements [7] makes it possible to obtain three-dimensional object reconstructions at a sub-nanometer resolution [8]. This approach not only yields excellent results in studying the structural features of relatively large objects, e.g., cells [9] and viruses [10], but also allows one to examine the interaction of the latter with bacteria [1] and visualize some proteins on the bacteriophage surface [10].

STANDARD SAMPLE PREPARATION AND MODIFICATIONS

At the first stage of sample preparation procedure, the electron microscope grids, which are usually coated with an amorphous carbon layer containing regular holes, are exposed to a glow discharge to impart their surface with hydrophilic properties. Then, 2–3 μL of sample solution are deposited on the prepared grid; the excess solution is removed with filter paper (except for a thin liquid layer left on the grid surface); and, using automated systems, vitrification is performed, i.e., flash-freezing of the sample in ethane or ethane/propane mixture, condensed at the liquid nitrogen temperature. This leads to the formation of a thin amorphous ice layer, the thickness of which can be controlled and is critical for the further experiment. For example, when studying bulk objects, e.g., cells, the ice layer should be sufficiently thick (up to several micrometers) to avoid their flattening and disruption of integrity.

The automated vitrification procedure allows one, on the one hand, to fix a sample in a close-to-native state and minimize radiation damage during data collection, and, on the other hand, to standardize the sample preparation procedure, thereby ensuring reproducibility of results.

The adhesion of eukaryotic cell systems to filter paper used during vitrification is often higher than the adhesion to the carbon substrate of the grid [11]. For such objects, cell systems are cultured directly on grid substrates [12]. However, some cells adhere to grids worse than to cultural plastics. To improve the adhesion properties, the grid surface is modified, for example, with poly-L-lysine [13]. In this case, gold grids are often used, which, unlike traditional copper or nickel grids, are nontoxic to cells [14].

LIMITATION OF THE OBJECTS SIZES

The maximum thickness of the sample, necessary to obtain a high-resolution three-dimensional reconstruction by cryo-ET, has certain limitations. As a rule, to detail well the microstructure of vitrified samples, their thickness should be no larger than the electron mean free path in amorphous ice, which is ~350 nm at a standard cryo-EM accelerating voltage of 300 keV [17, 18]. Because of this limitation, one cannot perform direct visualization of filling of most eukaryotic cells, thereby limiting the applicability of the technique to *in cellulo* experiments without additional sample thinning, and only allows for studying relatively thin external parts (lateral processes) on the eukaryotic cell periphery [17, 18] and some bacteria [19–21]. Thus, a question arises: how to perform additional sample processing in order to obtain sufficiently thin (electron-transparent) samples?

The use of the cryo-ultramicrotome [22] to obtain ultrathin sections of biological tissues [23], in the case of preparation of frozen cell sections [24], meets certain difficulties with preparing a block with cells, providing a necessary accuracy of diamond knife positioning, and transferring sections to the electron microscope grid under liquid nitrogen conditions. In addition, despite the high precision of diamond knives, mechanical cutting may introduce artifacts into the cell structure [25].

At present, some research teams optimize the sample thinning procedure using cryo-scanning electron and ion electron microscopy, specifically, cryo-FIB [28, 29], i.e., the modifications of a popular method of preparing specimens for transmission electron microscopy [28]. This approach consists in thinning the sample region of interest using Ga^+ ions with a gradual decrease in the accelerating voltage. The cryo-FIB preparation of thin sections of frozen cell systems ensures high accuracy of positioning the thinned cell region and minimizes the radiation damage of the sample when preserving a prepared section in the close-to-native state [29].

An alternative to the above-described sample thinning methods for studying cell systems *in cellulo* is their *in vitro* investigations [30–32]. In particular, after lysing intact cells, a cryo-ET study of individual cell filling structures can be carried out [33–35].

DATA COLLECTION

The development of new high-sensitive and fast direct electron detectors (DED) based on the complementary metal–oxide–semiconductor (CMOS) structure [36–38] made it possible to significantly improve the signal-to-noise ratio when obtaining experimental data and simultaneously reduce the exposure time, thereby minimizing the radiation damage and preserving the native structure of biological objects.

Since the formation of cryo-EM images is mainly affected by the phase contrast, a standard approach suggests obtaining images in a certain range of defocusing of the objective lens. However, simultaneously with the improvement of the signal-to-noise ratio at

low spatial frequencies, the increase in defocusing leads to deterioration of this ratio at high spatial frequencies, which makes it almost impossible to study particles with a molecular mass below 200 kDa [39]. An alternative way to form a phase contrast is to use phase plates, which are widely used in optical microscopy and, theoretically, may significantly improve the signal-to-noise ratio [40]; however, until recently, this way had not yielded any practical effect [43]. The occurrence of a new generation of phase plates [41] has significantly improved the contrast of experimental images, which is especially important for studying heterogeneous objects and complexes with moving parts [42]. The use of phase plates allows one to reduce defocus value in experiments, which allows to study proteins with at least 60 kDa molecular mass and positively affects the final resolution of three-dimensional reconstructions [43–46].

A significant increase in the experimental data contrast with the use of a phase plate was illustrated by an example of a magnetotactic bacterium and a neuron culture [47]: the cell membrane images obtained at a defocusing value of 8 μm are comparable in contrast with the images obtained (using a phase plate) under defocusing close to 0 μm; the spatial resolution was higher than 5 nm.

The cryo-FIB formation of electron-transparent sections in combination with the further cryo-ET experiments using a phase plate made it possible to carry out a large-scale study of the HeLa chromatin structure [48]: both megadalton complexes and macromolecules with a mass of up to 200 kDa, e.g., nucleosomes, were unambiguously identified. The latter became possible due to the sub-tomogram averaging with additional stages of the three-dimensional classification according to the template from [49].

THREE-DIMENSIONAL RECONSTRUCTION AND ANALYSIS OF EXPERIMENTAL DATA

Cross-Correlation Alignment

At the first stage of three-dimensional reconstruction, a cross-correlation alignment of the cryo-ET image stack is performed.

When studying objects frozen without additional sample processing (cell systems, bacterial cells, and viruses), an angular series of images is aligned using colloidal gold nanoparticles added to the investigated sample before vitrification [50]. In experiments with bulk intact samples prepared by the cryo-FIB technique, alignment of a tomographic stack is generally performed without gold fiducials. This can be done due to the use of a phase plate. However, a phase plate is sensitive to the charge of the obtained cryo-FIB lamella; hence, an additional conductive layer [29] should be deposited before the cryo-ET experiments.

Three-Dimensional Reconstruction

A three-dimensional reconstruction of a tomogram can be made using different algorithms, including weighted back projection (WBP) [51], simultaneous iterative reconstruction technique (SIRT) [52], etc. Tomograms reconstructed by the SIRT have a higher contrast as compared with the WBP-reconstructed ones. At the same time, the WBP technique more accurately transfers information about high spatial frequencies. Therefore, SIRT reconstructions are often used during data segmentation and annotation, while WBP is more appropriate for sub-tomographic averaging of a large number of identical macromolecules [53].

Data Annotation and Segmentation

At the next stage, the position of macromolecules on tomograms is determined using templates [54]. Segmentation and annotation of tomographic stacks are performed in a semi-automatic mode and generally requires application of several software packages. In particular, the TomoSegMemTV program [55] is often used for membrane segmentation, while the XTracing Module program in the Amira software package is applied for microtubules. Different macromolecules can be automatically positioned and aligned using their three-dimensional templates (PEET [56], EMAN2 [57], and Dynamo [58]). The templates can be based on the well-known three-dimensional structure of these macromolecules, determined by X-ray diffraction analysis or cryo-EM SPA [39, 59]. However, the significant heterogeneity of macromolecules and the presence of falsely chosen coordinates require subsequent iterative three-dimensional classification and averaging of small sections of a tomogram (the so-called sub-tomogram) containing individual macromolecules [17]. The development of machine vision has led to the occurrence of tools for automatic annotation and segmentation of many objects based on a small set of manually segmented tomographic sections [60].

High-Resolution Sub-Tomogram Averaging

The final spatial resolution of three-dimensional reconstruction depends to a great extent on the test sample thickness, alignment accuracy, angle step, and experimental angular range, as well as on the data processing algorithms in use. The spatial resolution of tomographic reconstruction is limited by a low signal-to-noise ratio and generally does not exceed 5 nm. However, in the presence of repeating structural elements in the test sample, the spatial resolution can be significantly improved using sub-tomogram averaging. To this end, the positions of investigated objects are determined on a reconstructed tomogram in the manual or automatic mode (IMOD [61]) and the corresponding sections of the tomogram are extracted (RELION [62]). The next step is to estimate the defo-

cus values for each image (CTFFIND4 [63]) and construct a three-dimensional model of the contrast transfer function. Then sub-tomograms are extracted, aligned, and averaged, thus allowing one to increase the signal-to-noise ratio and, consequently, the spatial resolution of an object [49], and final visualization of three-dimensional reconstructions is performed (UCSF Chimera [64]).

At present, a high (sub-nanometer) spatial resolution of macromolecules using cryo-ET has only been obtained *in vitro* for highly ordered structures, including virus capsids [8] or proteins laid in cylinders [68]. High spatial resolution (better than 4 Å) is provided using a great number (over 100000, which is comparable with the number of particles for the SPA experiment) of macromolecules for sub-tomogram averaging. In addition, it is necessary to satisfy the following conditions: dose-symmetric tilt-scheme for recording a tomographic series [65], an accelerating voltage of 300 keV, operation of Gatan K2xp CMOS detector in the counting mode, the use of an energy filter, and drift corrections for 10 subframes. The resolution can be additionally improved using new methods for correcting the contrast transfer function [66].

When studying more massive cellular systems, the spatial resolution is generally 20–40 Å. To date, a resolution of 11 Å has been obtained for the molecular architecture of protein aggregates in intact neurons [4].

CONCLUSIONS

The main approaches of modern cryo-electron tomography were considered. Cryo-ET is an extremely promising method for studying cellular systems in the native environment.

The complex use of such methods as vitrification and cryo-FIB preparation of electron-transparent samples, application of direct electron detectors to obtain experimental data, and improvement of contrast with the aid of a phase plate, along with rapid development of data processing algorithms, opens a window to *in situ* visual proteomics.

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