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Review

Molecular Electron Microscopy for Biology and Medicine

—— Overview ——

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Abstract

Electron Microscopy can now determine the structure of a biological macromolecule at a resolution that can resolve atoms. This technological development has allowed concerted efforts to obtain structural information by X-ray crystallography, NMR and other probe microscopes. These concerted approaches are used to determine the structures of multi-complexes of proteins, DNA, RNA and other biological macromolecules. However, such concerted approaches are still short to determine whole structures of more macroscopic organella or tissues.

On the other hand, rapidly improving electron tomography offers a means to determine whole structures of more macroscopic organella or tissues, although the resolution of the determined structures is not high enough to distinguish molecules. This article reviews and discusses how electron microscopy may determine macroscopic structures at molecular level to aid biology and medicine.

Key words: electron microscopy, cryo-electron microscope, electron crystallography, electron tomography, single particle analysis, supra-molecular complex, organelle structure, tissue

Introduction

Genetic technology now allows the study of biological and medical questions involving gene and genetic products, like proteins. Molecular structures of proteins, DNA, RNA or their macromolecular complexes have been investigated by X-ray crystallography, NMR and electron microscopy after purifying the related proteins in most cases. Purifying such macromolecules or complexes, however, comes typically after determining their involvements in biological topics, like functions or organelle/tissue structures. Determining the members, their roles and the interactions involved in a topic were a little too far away from structural analysis at the molecular level by these methods.

Electron microscopy has been a unique thread connecting the microscopic structures observed by optical microscopes and the molecular structures determined by X-ray crystallography or NMR. Electron microscopes were the only image-magnifying devices capable to resolve molecules. Electron microscopy that can determine the structures of macromolecules is called molecular electron microscopy. The original word 'microscopy' stemmed from the 'micron meter scale' world visualized by optical microscopes. The molecular world that molecular electron microscopy visualizes is at 'nano meter scale' and is therefore called nanoscopic world. The technological advance of molecular electron microscopy is rapidly bridging the conventional microscopic world, like organelles, tissues or cells, and the nanoscopic world, like biological macromolecules. The biological macromolecules are either gene-products or are produced by gene-products. The bridge between the two worlds is therefore a bridge between whole organism and gene. Thus, this technology is very much anticipated for biology and medicine after the genome project.

Electrons travel freely only in a vacuum. To use electrons for probing structures, a biological specimen must be placed in a vacuum

and also undergo electron irradiation. These conditions are usually too severe for biological specimens. Therefore, indirect methods of observing a mere replica or specimens stained with heavy atoms, like Uranium or Tungsten, were invented and adapted for electron microscopy. Indirect information, however, is often limited to the surface of a specimen and is susceptible to distortion or other artifacts. Another advance in electron microscopy comes from the cryo-technique, a technique that captures a specimen at an ultra low temperature typically lower than temperature of liquid nitrogen, −196℃, and then the specimen is observed in an electron microscope equipped with such ultra-low cold stage, cryo-electron microscope (Dubochet et al., 1982; Nickell et al., 2006; Chiu et al., 2006).

Image contrast in observing stained specimens is mostly created by scattering or absorbing electrons by heavy atoms around the materials of the specimen. They are noisy at the nanometer scale because the heavy atoms often forms aggregates at about 1–2 nanometer in diameter as a rough average during the drying process and the drying process often causes distortion of the specimens. Image contrast in observing unstained specimens is created by a phase shift in wave function of the electrons after their interaction with the materials of specimens. Generally, the contrast is so weak that accumulation and averaging of structural information are required. In the case of crystals or multiply symmetrical specimens, like virus, accumulation and averaging of structural information can be done by following the repetition of molecules in the specimens, resulting in a resolution as high as that of X-ray crystallography (Fig. 1). Thus, this technique is often called electron crystallography. In the case of low or no symmetrical specimens, accumulation and averaging of data are done computationally by comparing particles (proteins or their complexes) one another and aligning their orientations (Fig. 1). This technique is called single-particle analysis, indicating the detailed analysis of every single particle. Although confusing, single particle analysis assumes accumulation of numerous particles after the actual analysis of every single particle (Frank, 2006b).

In the following sections, I first describe electron crystallography and then single particle analysis. Finally I will describe

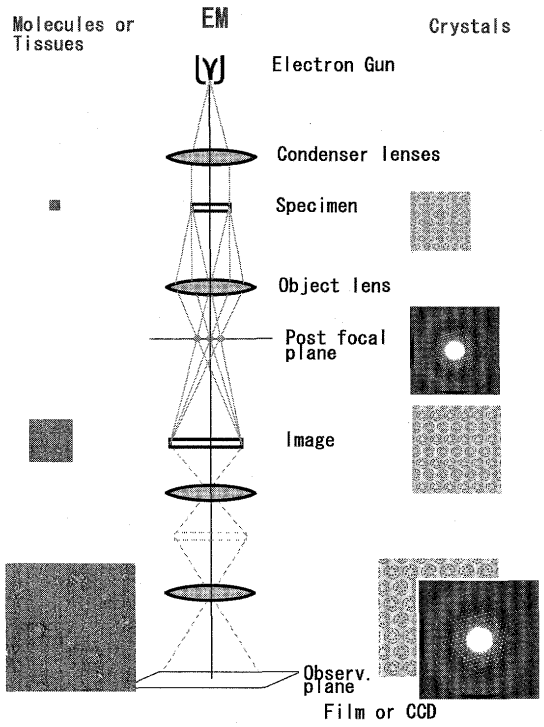

Fig. 1 Schematic diagram of transmission electron microscope (middle) for observing molecules or tissues (left) or crystals (right). An electron microscope typically has an electron gun, two or three condenser lenses, specimen holder with or without a cooling device, object lens, a few intermediate lenses and one or two projector lenses on an observing screen, film-camera and/or CCD-camera, similar to optical microscope. To describe typical observations, a post focal plane and image for object lens are shown

electron tomography, in which many images of a single particle, organelle or tissue are obtained by tilting it at various angles. Then, I will propose that a structural analysis of organelle and tissues that may resolve molecular details.

Electron crystallography

As indicated briefly, the contrast of images from unstained-specimens is generally so weak that the accumulation of information is required. If molecules or complexes are arranged regularly as one may find in crystals, the accumulation of structural information can be achieved by following such regularity. A structural map, corresponding to the density of the molecule in three dimensions, at 7 Angstrom was obtained in 1975 by analyzing images of the two-dimensional crystals of a membrane protein, bacteriorhodopsin, and

combining these data with their diffraction data (Fig. 1 right side) (Henderson et al., 1975). The work demonstrated the structure of 7 alpha helices that span a membrane, which was for the first time detected in this work but is now a well known finding for many important receptors. A single two-dimensional (2D) crystal (0.5 μm in diameter) contains about 17000 molecules. A crystallographic analysis consequently sums up the data from these molecules. The molecules were aligned by crystal formation. Determining crystallographic parameters was almost sufficient because the summing of structural information over the entire crystal was achieved by simple calculation. The drawback was that structural information in the direction perpendicular to the surfaces of two-dimensional crystals was poor and the length of helices was short and the helices were not connected at the time. Most of the drawbacks have been overcome, and the map has now reached 3 and then 2.4 Angstrom in practically all directions with all connections of 7 alpha helices well resolved (Kimura et al., 1997).

The analysis of electron crystallography is briefly presented (Fig. 2). With an electron microscope, we can observe both diffraction and image of a crystal (Fig. 1). Though it is possible to observe diffraction using 3D crystals, it is far easier to accumulate data from 2D crystals for instrumental reasons; it is difficult to devise a precise multi-axial goniometer as X-ray crystallography requires within electron microscopy for one reason. Observation of phases by image analysis is unique to electron crystallography. Only a single diffraction or image at a particular orientation is observed from a single crystal due to damage by electron irradiation. Therefore, data from many crystals are combined (Fig. 2a). In this figure, data from more than 600 crystals were merged. The crystal sizes were generally 2 times larger (c. a. 70000 molecules) and therefore about 40 million molecules contributed to the whole data set.

Due to the limit of tilting two-dimensional crystals against the direction of the electron beam, the data could be obtained up to 70 degree in this case (Fig. 2b). However, the missing data comprised only 5% of total data. It turned out to be not so significant in this case. If there is a greater lack of data, the missing data will cause serious problems in the final map. This problem is called 'missing

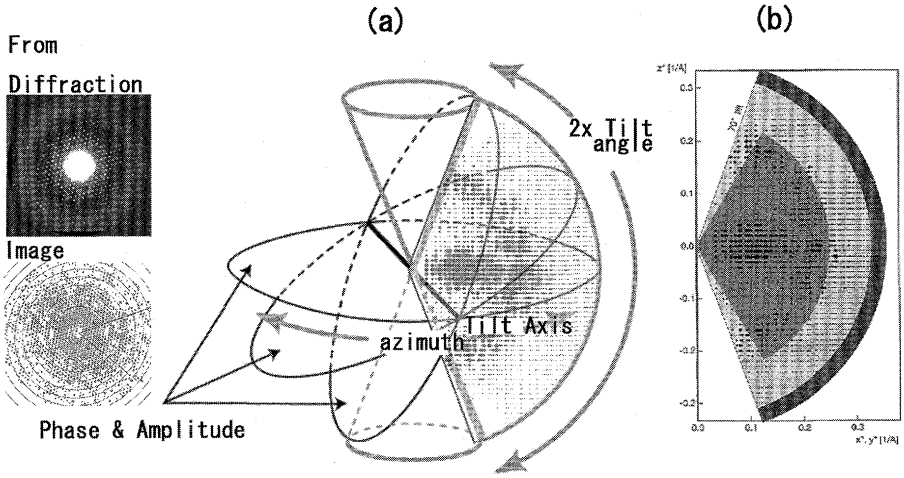

Fig. 2 In the case of a two-dimensional crystal of a membrane protein, bacteriorhodopsin, images were Fourier transformed to determine phases and the corresponding electron diffraction was used to determine the Fourier amplitude. By combining amplitudes and phases, the original 3D map was calculated. Only a single diffraction or a single image at a particular orientation was taken to avoid specimen damage from electron irradiation. Therefore, data from many crystals are combined. In this figure, data from more than 600 crystals of bacteriorhodopsin were merged. More than 40 millions of bacteriorhodopsin contributed to the final map. Each diffraction and Fourier phases correspond to a plane in the Fourier space (a). Due to limits in tilting two-dimensional crystals against the direction of electron beam, the data could be obtained only up to 70 degree in this case (b). However, the missing data comprised only 5% of total data and were not highly significant in this case. The lack of data could sometimes causes problem, so called the 'missing cone problem' as the missing data zones form the shape of cone in Fourier space (a)

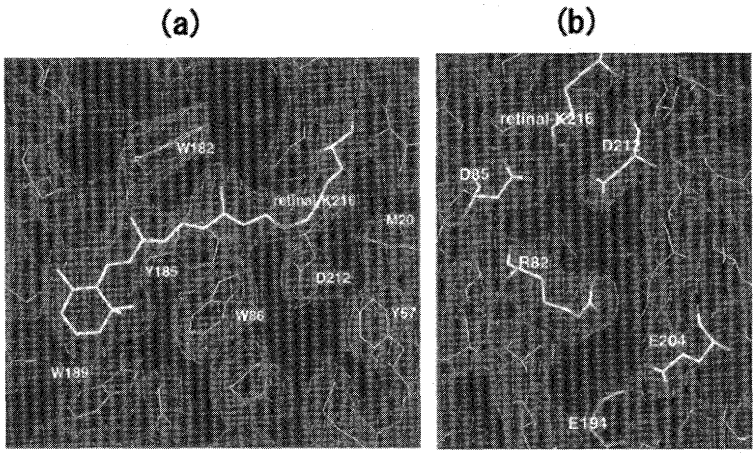

Fig. 3 Bacteriorhodopsin model and map at 2.4 Angstrom. Bacteriorhodopsin is a membrane protein that carries retinal as a chromophore and pump protons using absorbed light energy. It consists of 7 transmembrane alpha helices. Retinal and surrounding amino acid residues (a) are clearly visible. The retinal binding lysine and acidic residues in the proton pathway are shown (b)

cone problem' as the missing data zone are in the shape of cone in reciprocal space (Fig. 2a).

The final map at 2.4 Angstrom obtained purely by electron crystallography well resolve the retinal (a chromophore of bacteriorhodopsin) and the surrounding amino acid residues (Fig. 3a). All the essential amino acid residues and some water molecules were observed. It should be noted that electron crystallography demonstrated the full structure of bacteriorhoidopsin for the first time but the same structure was later confirmed by X-ray crystallography. Thus, the structural analysis by electron microscopy was considered comparable to other methods. Another important message is shown in Fig. 2b, which represents data accumulation in the X*, Y*-Z* plane (Fourier space, Z* axis corresponds to the real space axis perpendicular to the two-dimensions; i. e. membrane). The inner dark shaded zone is a data zone where practically all phase data remain almost unchanged from the observed phases after refinements. The light shaded zone between 5 and 3 Angstrom) required phase improvements. The phase data in the very dark zone were largely ignored, though some were obtained. In other words, phases from images lower than 5 Angstrom are very reliable and phases between 5 and 3 Angstrom require caution.

Since electron crystallography produce data contributed from many particles, 15000–70000 molecules from a single crystal of bacteriorhodopsin, it was possible (and necessary for the result described above) to assess the maximum dose that preserve structures for needed resolutions. Obtaining good images require drift-free while exposed to electron irradiation for about 1–2 seconds. The maximum dose was 20–25 electrons/A^2 for 3 Angstrom resolution and 40–50 electrons/A^2 for 6 Angstrom at −269℃ (4 Kelvin, liquid helium temperature). About 10 electrons/A^2 was the maximum dose for 6 Angstrom resolution at −196℃ (liquid Nitrogen temperature). Liquid helium stages (side entry types) seldom go below 10 Kelvin. At 10–20 Kelvin, images at 20–25 electrons/A^2 doses seldom reached higher resolution than 5 Angstrom. Electron diffraction, on the other hand, tolerates drift and therefore diffraction data were recoded at longer exposure times, 15 sec–30 min, under weak beam intensities. With larger crystals (about 2 μm, containing c. a. 280000 bacteriorhodopsin) at the 30–40 electrons/A^2 doses, the diffraction data went beyond 2 Angstrom.

Single particle analysis

Electron crystallography now offers very nice high resolution comparable to X-ray crystallography. Preparing crystals for electron crystallography, however, is not easy. Two-dimensional crystals may adhere to biological membrane proteins, but just purifying membrane proteins to the quality necessary

for crystallography is very difficult and their two-dimensional crystallization is still in the realm of fine art. Bacteriorhodopsin was a special case because it forms two-dimensional crystal in the nature.

Single particle analysis, however, deals with separate particles, like molecules or their complexes. The method sums up structural information from many particles to obtain the structure of a particular protein or complex in three dimensions by taking images of every single particle of the molecule/complex then analyzing these images. Since there are excellent text books describing the principles and procedures for this method (Frank, 2006 b), here and I will describe only outlines of the analysis (see Fig. 4) and a few details that may be relevant to a later part of this review.

Specimens are usually negatively stained for the initial structural study. Though the information from stained specimens is limited to the exterior of a molecule/complex, namely the outline profile of the molecule/complex, the achievable resolution reaches 15–20 Angstrom. The actual resolution may depend upon the quality of images and the number of particles accumulated and analyzed. To observe a rough shape of a very large particle (molecular machinery made from proteins), roughly 100 to 5000 particles are accumulated to produce structural 3D maps at resolutions 20–50 Angstrom. To have parts within a

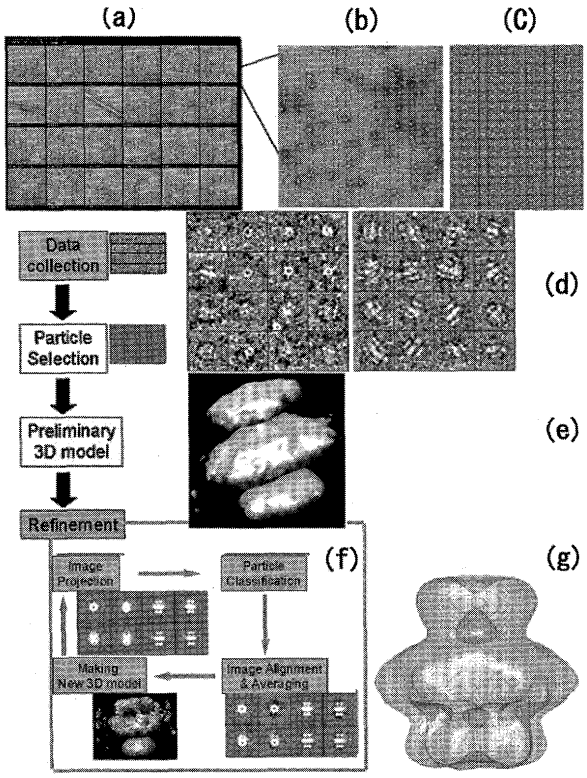

Fig. 4 Schematic diagram of single particle analysis. A browse of many images (a), each of which carries many particles (b). Images of the particles were selected (c), classified and averaged into various class images of particles (d). Then, (e), a preliminary 3 dimensional model was calculated from such class averaged particles after determining orientations. Angles of orientations can be determined by finding common features among images. Ideally, only unique orientation can account for various images taken at various angles. Noises, contaminations, and conformational changes often cause mistakes in determining the orientations. From the preliminary 3D model (e), new projection images are calculated at various evenly distributed angles for the directions. Such new projection images are used to classify original particles, new class averages were recalculated and then a new 3D model was calculated. After such refinement cycles, a final 3D model is obtained (g)

particle distinguished. 1000–20000 particles are accumulated at resolutions 15–25 Angstrom using stained specimens. For a resolution higher than 15 Angstrom, cryo-conditions are required. Though the signal contrasts from cryo-specimens are very much weaker than those from stained specimens, well accumulated maps can reach a resolution of around 5 Angstrom, and may even reach a higher resolution, with direct internal information of specimens being obtained. To obtain a structural map at a resolution better than 10 Angstrom, 100000 or more particles need to be accumulated from cryo-specimens.

Once data are accumulated (Fig. 4a), particles are selected manually or automatically (Fig. 4b, 4c). Automated selection of particles is desirable, but manual selection is better at the beginning of selection. The selected particles are then classified, and the images that belong to each class recognized as identical are averaged (Fig. 4d). If classes are from an identical three dimensional (3D) object, any two of different classes share a common line; take a projection of a two-dimensional image into one dimension in a direction, at least one such projection from two images should coincide (within a limit of resolution under noise levels). Thus, the class averages are compared. If there are enough classes, a preliminary 3D map can be calculated (Fig. 4e) if the angle-distribution is known. The number depends on the distribution of the projection in three-dimensional space and the quality of images. Without degeneracy and crowded details, only three classes might function like an architectural blueprint. In reality, about 50 classes are initially desirable for biological specimens. If there is symmetry in the specimen, the number of classes can be reduced considerably. The angles that specimens may take in space are either calculated from images or determined experimentally. As noted already, two different projection images (2D) share a common line. In the process of determining a common line, an angular relationship is determined. More than two such combinations should decide the mutual angular relationships. The calculation, however, might lead to a labyrinth. Experimental methods often require taking two images before and after tilting a specimen at a known amount of angle. Previously, a preferential orientation of particles was required but now such restraint has been lifted due to the use of a combined method to determine the angles. Introducing known angles can reduce error and a preferential orientation is often desired.

If a preliminary 3D map is obtained, new projection images can be calculated at various evenly distributed angles for the directions (called reprojection). These reprojected images are used to classify original noisy images. After the re-classification, a new 3D map is calculated from the new class-averages (Fig. 4 f). After several cycles of such refinement, a final model is obtained (Fig. 4g).

When the final map is obtained, the map is usually compared with structure(s) determined by other methods if any or calculated models (Fig. 5). In Fig. 5, the fitted model in the middle part was determined by X-ray crystallography from genetically truncated specimens. The model fits very well to the details of the 3D map obtained by single particle analysis. The top part is fitted with a model and roughly both are the same size. The bottom part differs from the structure determined by X-ray crystallography for the truncated specimen for this part. The reason of the difference is not known yet but generally the difference often reflects conformational change in such case.

As the single particle analysis is now rapidly being improved, the secondary structures and their conformational changes have started to be determined reliably.

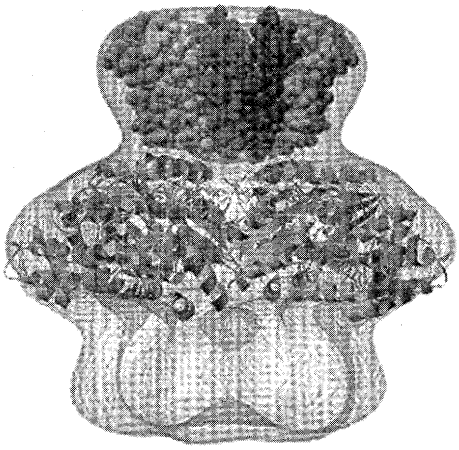

Fig. 5 The calculated model was then compared with structures determined by other methods, such as x-ray crystallography, NMR, and purely calculated models

Electron tomography

Single particle analysis and electron crystallography take single or at most two pictures at a time and the reconstitution of a 3D map is done with many data from many particles at different tilting angles. In this case, determining tilting angles is one of the major efforts in the image analysis. However, electron micrographs can be reconstituted in three dimensions from a series of images taken at known tilted angles (Fig. 6), every 1–5 degrees between −60 and +60 degree typically. This method is called electron tomography just like 'computer aided x-ray tomography' or MRI used in hospitals (Nickell et al., 2006; Frank, 2006a). To achieve such observation, the axis of rotation must be at the center of the area and the instrumental parameters for taking micrographs, such as focus, astigmatism-correction etc, must be consistent in all images. Thus the major efforts of this method are made by the manufacturers of electron microscope. This method requires 25–120 exposures of the same specimen and therefore the total amount of electron irradiation is very large. For instance, 10 electrons/A^2 for 50 images will total 500 electrons/A^2. Because such dose is too much for cryo-specimens, the technique has been applied mostly to stained specimens or tissues at relatively low resolutions, 50–100 Angstrom.

As a hybrid of the negative staining method and cryo technique, cryo-negative staining method has been investigated for observing biological specimens (Fig. 7c) (El-Bez et al., 2005). In this method, specimens are frozen captured in a medium containing heavy atoms. The heavy atoms create high contrast at the boundary of a particle and the medium. Since the structure is kept frozen while the micrographs are taken, the distortion should be small. As far as the medium structure is kept intact (frozen locally), the boundary should be intact. Here comes my proposal for electron tomography.

To minimize damage by electron irradiation, I have been taking images intermittently; images are taken not at one shot but at many times at intervals. In the combination of ultra cold stage, the rise of temperature during exposure and accumu-

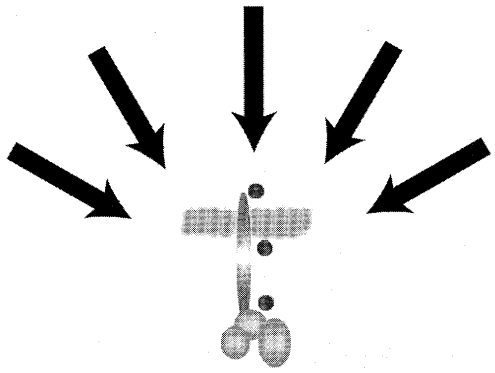

Fig. 6 Schematic diagram of taking images for electron tomography. Images are taken at various tilt angles of the specimen-stage. In this figure, various beam directions are shown to depict the tomography that takes images of an object from various directions. Black dots on the schematic specimen stand for gold particles labeled on the object for identity and also for determining locations of particular amino acid residues labeled with the gold particles

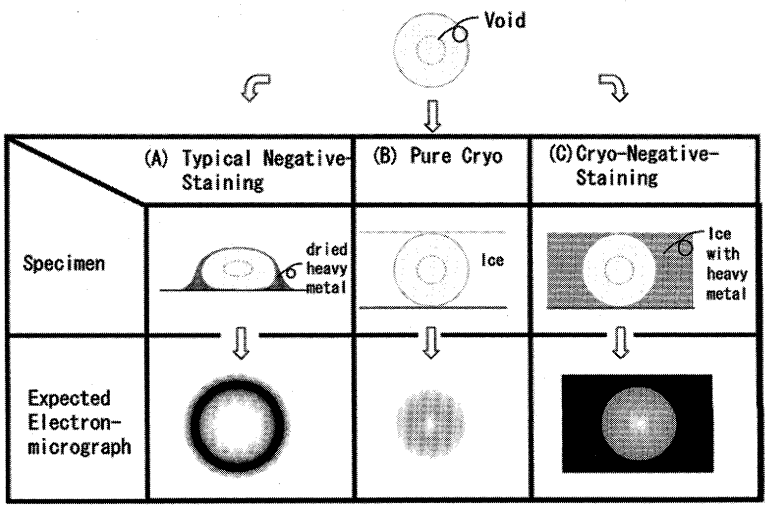

Fig. 7 Difference between stained (A, C) and unstained (B, =pure cryo) specimens. Typical staining process dry specimens and the contrast occurs near the edge of specimens (A). In this case, biological specimens are often distorted, which often causes the resultant structure to be inaccurate and sometimes misleading. Pure cryo condition captures specimens in the physiological buffer by rapid-freezing technique and the specimens are observed in electron microscope equipped with cold stage and devices that optimize irradiation dose. In the case of cryo-negative staining method (C), specimens are frozen captured in medium containing heavy atoms. The heavy atoms create high contrast and yet the internal structure remains undistorted

lation of charges at the irradiation area are divided into small portions in single exposure and are dissipated during the intermittent period. This concept is supported by hard evidence from electron diffraction. In measuring electron diffractions intermittently, the decay of diffraction spots at high resolutions was traced. The result showed a very good cryo-preservation. Similar preservation was observed for images as well (Fig. 8). In this case, the same area of the same specimen was recorded at a liquid helium temperature (−269℃). Cross correlation of each image with the first image was plotted for the order of taking pictures. Under good conditions, cryo-negative stained specimen could yield the same high correlation with the first image after 60 exposures. A pure cryo image always shows a rapid decrease in the correlation depending on the irradiation dose.

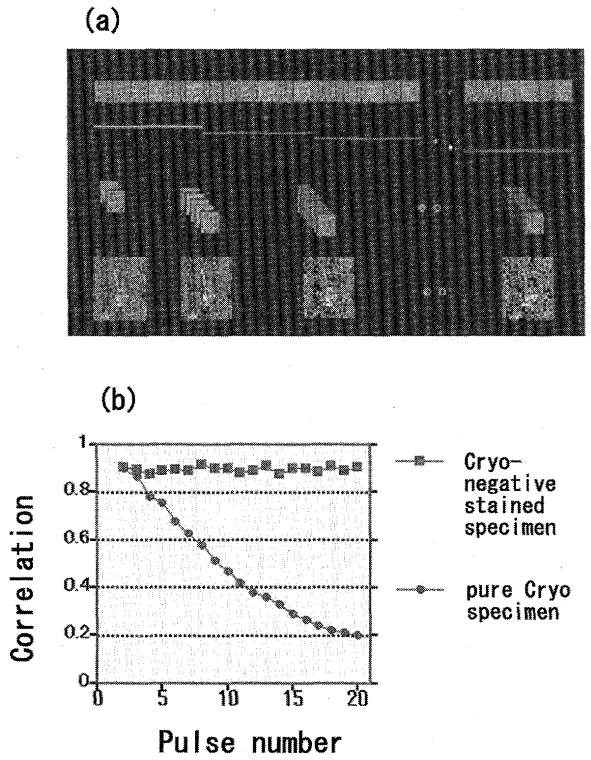

Fig. 8 Schematic diagram of taking multiple images at the same area of the same specimen with optimized irradiation dose (a). The multiple images are stored separately and are used for image analysis afterward. If a single image is not good enough for a particular analysis, several images could be averaged and characterization of the average is done to determine parameters for further image analysis. (b) Comparison of pure cryo and cryo-negative stained specimens. In this case, the same area of the same specimen was recorded at liquid helium temperature (−269℃). Cross correlation of each image with the first image was plotted for the order of taking pictures. In this case, cryo-negative stained specimen demonstrated the same high correlation with the first image after 60 exposures. Pure cryo image always shows a rapid decrease in correlation depending on the irradiation dose

I am proposing electron tomography under similar cryo-negative conditions. The outer shape will remain unchanged, thus producing a good outlier map in three dimensions. After obtaining the outlier map as a guide for alignment of particles, the initial images of the intermittent series should keep information at high resolutions inside the outlier shape. The accumulation of such initial images with good aligning parameters should ultimately produce a good 3D map, even comparable to that obtained with electron crystallography. Under a cryo-negative condition, heavy atoms are evenly dispersed because the medium is not dried. About 6 Angstrom resolution was reported for that measurement. At 6 Angstrom, major secondary structures (alpha helices and sheets) should be distinguishable. With such alignment parameters, I believe a 3 D map at 3 Angstrom is a possible target.

If we can observe organelle and tissues with electron tomography under such high resolution, genetic and structural information would linked and become available for biology and medicine.

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