Three-dimensional distributions of elements in biological samples by energy-filtered electron tomography

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Abstract

By combining electron tomography with energy-filtered electron microscopy, we have shown the feasibility of determining the three-dimensional distributions of phosphorus in biological specimens. Thin sections of the nematode, Caenorhabditis elegans were prepared by high-pressure freezing, freeze-substitution and plastic embedding. Images were recorded at energy losses above and below the phosphorus L$_{2,3}$ edge using a post-column imaging filter operating at a beam energy of 120 keV. The unstained specimens exhibited minimal contrast in bright-field images. After it was determined that the specimen was sufficiently thin to allow two-window ratio imaging of phosphorus, pairs of pre-edge and post-edge images were acquired in series over a tilt range of $\pm 55^\circ$ at $5^\circ$ increments for two orthogonal tilt axes. The projected phosphorus distributions were aligned using the pre-edge images that contained inelastic contrast from colloidal gold particles deposited on the specimen surface. A reconstruction and surface rendering of the phosphorus distribution clearly revealed features 15–20 nm in diameter, which were identified as ribosomes distributed along the stacked membranes of endoplasmic reticulum and in the cytoplasm. The sensitivity of the technique was estimated at $<35$ phosphorus atoms per voxel based on the known total ribosomal phosphorus content of approximately 7000 atoms. Although a high electron dose of approximately $10^7$ e/nm$^2$ was required to record two-axis tilt series, specimens were sufficiently stable to allow image alignment and tomographic reconstruction.

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1. Introduction

Electron tomography is becoming a well-established and important biophysical technique for determining the three-dimensional structure of macromolecular assemblies and subcellular organelles [1–6]. To obtain ultrastructure that is well preserved, cells or tissues are typically prepared by cryofixation, freeze-substitution, plastic embedding, sectioning and staining. When a suitable specimen has been prepared, a series of electron micrographs is recorded over a range of tilt angles.
from a region of interest. Then by using a back projection algorithm, it is possible to compute the three-dimensional structure of the object [7]. If the specimen is stained with heavy metals to produce amplitude contrast in the bright-field electron micrograph, the tomographic reconstruction corresponds to the distribution of stain that becomes bound to structures within the cell. Electron tomography can also be applied to unstained frozen-hydrated specimens by defocusing the objective lens of the microscope to provide phase-contrast imaging [8–12].

Until now, there have been no reports of tomography based on electron spectroscopic imaging for determining the three-dimensional distribution of specific elements within biological structures. Recently, however, there has been progress in electron spectroscopic tomography of inorganic materials [13,14]. One of these studies reported the three-dimensional iron distribution of inorganic inclusions occurring in bacteria [13], but no attempt was made to analyze the organic components in the sample. With advances in energy-filtered electron microscopy (EFTEM), including the availability of efficient charge-coupled device (CCD) detectors [15], it is reasonable to consider the feasibility of performing elemental tomography on biological components within cells [16]. For example, phosphorus is associated with phosphate groups of nucleic acids, and significant sulfur concentrations are associated with proteins that contain high amounts of the amino-acids cysteine and methionine [17–19]. One faces several challenges in attempting to map elements in three dimensions. First, biological specimens generally contain relatively low concentrations (typically <1 at. %) of even the major elements like phosphorus and sulfur. Therefore, special attention must be given to subtracting the core edge signal from the background in the energy loss spectrum [20–23]. Second, the increased effective specimen thickness at high tilt angles could further limit the reliability of the background subtraction, particularly for small ratios of core-edge signal to background intensity that occur in these specimens [24]. Third, biological specimens are susceptible to beam damage and it is unclear whether an entire image tilt series can be acquired without losing registration between the individual images in the series [25]. Finally, as for conventional biological electron tomography, it is necessary to deposit clearly visible markers on the specimen to align successfully the individual images in the tilt series [26]. In the case of elemental tomography, these markers must be observable in the energy-filtered images around the core-edge of interest.

To test the feasibility of performing elemental tomography on biological specimens, we have recorded phosphorus maps from unstained thin sections of the nematode, Caenorhabditis elegans, which has been studied extensively by molecular geneticists because it is one of the simplest multicellular organisms [27]. Because of its small size (diameter ca. 100 μm), C. elegans can be frozen at high pressure to provide excellent preservation of its structure while still allowing preparation of thin plastic sections that are suitable for energy-filtered imaging [28].

2. Experimental method

2.1. Specimen preparation

To obtain unstained sections for elemental mapping in the energy-filtering electron microscope, C. elegans, in 15% sucrose as a cryopreservative, was deposited into a 2-mm inner diameter specimen carrier and was frozen at a pressure of approximately 2100 atm in a BALTER HPM-010 high-pressure freezing machine [28,29]. The frozen blocks were processed at low temperature in a Leica EM-AFS freeze-substitution system using a solution of acetone with 0.2% ethylene glycol-bis-succinimidyl succinate. The fixation and removal of water were carried out below the ice re-crystallization temperature of −70°C. After freeze-substitution for 3 days, the specimens were gradually warmed up to room temperature and embedded in Epon-Araldite by graded exchange of the acetone. Specimens were polymerized in 100% resin by heating to +60°C for 2 days, and were subsequently sectioned to a thickness of about 70 nm with a Leica Ultracut E ultramicrotome. The unstained sections were
mounted on copper EM grids for analysis in the energy-filtering electron microscope. To apply gold particles used as fiducials for alignment of the tilt series, the grids were glow-discharged in air for 20 s. Then 5 μl of 10-nanometer immuno-gold conjugated to goat anti-rabbit IgG (BB International) was applied to the sections and left to adsorb for 1 min, after which the grids were washed three times in de-ionized water to remove excess reagent.

2.2. Energy-filtered imaging and EELS

Elemental maps were obtained using a CM120 transmission electron microscope (FEI/Philips Electron Optics) equipped with a GIF100 post-column imaging filter and a spectrum-imaging system (Gatan Inc.)[30,31]. To optimize contrast in the thin unstained sections, a 20-μm diameter objective aperture was inserted. Phosphorus distributions were obtained by applying the two-window ratio method or three-window method. In the two-window ratio method, it is assumed that background below the P L$_{23}$ edge has the same shape across the entire image, and the phosphorus distribution can therefore be obtained from the ratio of the post-edge image and the pre-edge image[32]. In the three-window method, two pre-edge images are used to estimate the post-edge background intensity by using an inverse power law $I \propto E^{-r}$, where $E$ is energy loss and $r$ is the exponent of an inverse power law behavior[23]. The two-window ratio method is known only to be valid for specimens that have a thickness $t$ less than about half of the total inelastic mean free path, $\lambda_{in}$[19,32]. The relative specimen thickness was determined by using the imaging filter in spectrum mode to record the low-loss spectrum over a 100 eV energy range. Spectra were transferred into the Gatan EL/P program and the relative thickness was determined from the relation, $t/\lambda_{in} = \ln[I_{tot}/I_0]$, where $I_{tot}$ is the total intensity in the spectrum and $I_0$ is the zero-loss intensity [23]. The mean relative thickness $t/\lambda_{in}$ of the plastic sections studied in this work was measured to be 0.30 and this value only varied by 10% over a typical image region. The output of the 1024 × 1024 pixel cooled CCD array detector was binned to 512 × 512 pixels to improve the signal-to-noise ratio. Images were acquired at 120-kV beam voltage and processed by means of Digital Micrograph software (Gatan Inc). Energy-selected images were acquired in the vicinity of the P L$_{23}$ edge using a 15-eV slit width with a pre-edge window at 120 eV and a post-edge window at 157 eV for ratio imaging. To apply the three-window mapping procedure, images were recorded at 90 and 120 eV below the P L$_{23}$ edge and at 157 eV above the edge. The acquisition time for each energy-selected image was 4 s. The software automatically corrected for specimen drift by applying a cross-correlation algorithm between successive exposures.

EFTEM does not easily provide a means of examining the energy loss spectrum from regions of the specimen that are a few nanometers in size. Since the usefulness of electron spectroscopic tomography depends on the reliable extraction of relatively weak core-edge signals, we have employed the technique of electron energy-loss spectrum-imaging in the scanning transmission electron microscope (STEM) to assess the reliability of the EFTEM two-window ratio imaging. In STEM spectrum-imaging, a finely focused electron probe is digitally scanned across the specimen and a parallel-recorded spectrum is collected at each pixel[33,34]. Spectrum-images were acquired at a beam energy of 100 keV using a dedicated VG Microscopes HB501 scanning transmission electron microscope (STEM) equipped with a Gatan Enfina electron energy-loss spectrometer (EELS), and controlled by Gatan Digital Micrograph spectrum-imaging software, as previously described[35,36]. STEM analyses were performed on the same specimen that was imaged by EFTEM; regions about 400 nm in width were imaged with dwell times of 0.2 s per pixel and a pixel size of 5 nm. The collection semi-angle referred to the specimen was 20 mrad and the probe current was adjusted to approximately 0.5 nA. Distributions of phosphorus were obtained from the analyzed regions by fitting an inverse power function below the P L$_{23}$ edge at each pixel and by subtracting the extrapolated background above the core edge. Features in the resulting phosphorus map could be selected and the
corresponding energy loss spectrum inspected. In this way, spectra were extracted from individual ribosomes and the STEM–EELS signal compared with that obtained by the EFTEM imaging.

2.3. Electron tomography

A series of energy-filtered images was collected over an angular tilt range from $-55^\circ$ to $+55^\circ$ with increments of $5^\circ$ at energy-losses above and below the P L$_{23}$ edge at 132 eV. The energy-selecting slit was adjusted to 15 eV and image pairs were recorded at 157 and 120 eV. The sample was then removed from the holder, rotated by $90^\circ$, and a second tilt series was recorded in the tilt range $-55^\circ$ to $+55^\circ$ with the same $5^\circ$ tilt increment. The angular range was limited by the geometry of the specimen holder, which obstructed the electron beam at high tilts.

The tomographic double-axis tilt series was reconstructed using the IMOD software program from the University of Colorado [37,38]. The volumes of the two orthogonal tilt series were reconstructed separately, after which their mutual orientation was determined by a 3-D cross-correlation method. The Fourier transforms of the two volumes were then combined and back-transformed to give the final 3-D volume.

For each single-axis tilt series, the 3-D volume was calculated using a backprojection algorithm, which assumes that the projections of tilted images arise from rotations about a fixed tilt axis. To perform the backprojection, the images recorded at different tilt angles have to be brought into register, and the real tilt differences between the subsequent images have to be determined. The tilted images are first aligned pairwise by cross-correlation using the nominal tilt angles, resulting in a series of the so-called pre-aligned images. Gold particles are then selected on these pre-aligned tilts, either by picking the fiducials manually on each image in the tilt series, or, in certain cases the algorithm can locate the fiducials automatically. For automatic location of the fiducials, the gold particles have to be identified on the zero-tilt image, and the program finds the particles in all the other images in the tilt series. The program analyzes the particle positions and recommends changes in tilt angles and tilt axes for each image, which would make the particles lie in a horizontal plane. This step produces a set of corrected tilt angles and tilt-axis positions for each image. Using the parameters (shifts and rotations) of the pre-alignment, and the corrected positions of the tilt-axes, the final aligned tilted images are calculated from the original tilts. The 3-D volume is then calculated from the final aligned images and from the corrected tilt angles.

To find the mutual orientation of the two volumes reconstructed from the two orthogonal tilt series, the coordinates of the fiducials in each tilt series are used to determine the 3-D rotation and distortion between the two tomograms. This initial alignment is refined by 3-D cross-correlations of small patches extracted from both volumes. The final match between the volumes is generated by a series of linear transformations calculated from the displacements of the individual patches. The density between the two volumes is matched before their Fourier transformations are calculated.

Visualization of the 3-D volume is provided by the Amira software package (TGS, http://www.amiravis.com/). The best visualization of the spatial organization of phosphorus was achieved using volume rendering. Intensity values were thresholded to eliminate the background, and MPEG movies were produced by the MovieMaker module in Amira.

3. Results

A typical region of an unstained, freeze-substituted and plastic-embedded cell of *C. elegans* in Fig. 1a shows negligible contrast in the bright-field image except for the presence of dense gold particles used for alignment. The specimen thickness was measured as $0.30 \pm 0.03 \mu m$ from low-dose analyses of spectra such as the one presented in Fig. 1b. The uniformity of the specimen thickness is shown again by the phosphorus pre-edge image in Fig. 1c recorded at an energy loss of 120 eV. A line profile across the center of this image in Fig. 1d shows only a $\pm 5\%$ variation in intensity. At a tilt angle of $60^\circ$, the effective thickness in the beam
direction increases to about 0.6\(\lambda_{in}\), under low dose conditions. However, the effective specimen thickness for electron tomography was reduced by pre-irradiation with an electron dose of approximately \(10^5\) e/nm\(^2\), which was applied to stabilize the specimen prior to recording the tilt series. This produced a rapid loss of approximately 20% of the specimen mass per unit area, as judged by the decrease in inelastic signal. The effective thickness at 60° tilt was therefore only about 0.5\(\lambda_{in}\), which is thin enough to satisfy conditions for two-window ratio mapping in the EFTEM [32]. Since there did not appear to be any appreciable shrinkage in the specimen plane by pre-irradiation, it is likely that the plastic sections suffered partial collapse in the \(z\)-direction, which one might expect to be noticeable in the tomographic reconstruction.

To check the strength of the phosphorus signal from structures within the same unstained plastic section that were analyzed by EFTEM, 1340-channel spectra were acquired using the complementary technique of STEM–EELS. Extracted energy-loss spectra from a spectrum-image are shown in Fig. 2a for a region of cytoplasm and in Fig. 2b for a phosphorus-containing feature that we later identify as a ribosome. For each spectrum, the background below the phosphorus L\(_{2,3}\) edge was fitted by minimum least-squares to an inverse power law and extrapolated into the post-edge region, as shown in Fig. 2. No phosphorus was detected in the cytoplasm but a clear phosphorus signal was detected in the ribosome with a signal-to-background fraction of approximately 0.1 for an energy band 15 eV wide centered at...
157 eV, i.e., 25 eV above the P L₂₃ core edge energy of 132 eV. The good background fit in the pre-edge region of the STEM energy-loss spectrum, and the observed characteristic shape of the subtracted phosphorus L₂₃ edge [23], support the validity of using the two-window ratio mapping approach to image phosphorus by EFTEM.

The applicability of the EFTEM mapping approach for phosphorus is confirmed in Fig. 3, where a typical phosphorus two-window ratio map and phosphorus three-window map from the same specimen area show similar contrast. As expected, the signal-to-noise ratio is higher in the ratio map than in the three-window map, for which there is an added statistical uncertainty in the background estimation caused by two parameters instead of one being used to fit the background shape of the energy loss spectrum [32]. The two-window ratio method for mapping elements in thin specimens is valid if the shape of the background (i.e., the exponent of the inverse power law below the phosphorus edge) does not change appreciably across the specimen region. In this case the signal $S$ is equal to the post-edge intensity $I_{\text{post}}$ minus a constant $k$ times the pre-edge intensity $I_{\text{pre}}$, which gives

$$S = I_{\text{post}} - kI_{\text{pre}}.$$

If the ratio image $R$ is defined as $I_{\text{post}}/I_{\text{pre}}$, then $S = (R - R_0)I_{\text{pre}}$.

The phosphorus signal $S$ is therefore obtained from the ratio image $R$ by first subtracting a constant $k = R_0$, corresponding to the measured value of ratio image in the plastic section where the phosphorus signal is zero, and then multiplying by the pre-edge image intensity.

To estimate the electron dose for each energy-filtered image used in the phosphorus imaging, an unfiltered 1024 × 1024 pixel bright-field image was recorded under the same condenser lens excitation with an exposure time of only 0.1 s. Each pixel contained approximately 2500 counts, which corresponds to $10^5$ incident electrons in a 4-s exposure, since the image intensity of the CCD camera had previously been calibrated as approximately 1 count per incident electron.
Fig. 3 shows that the 10 nm gold markers are evident in the pre-edge images as bright features due to their increased inelastic scattering cross-section. However, at certain angles in the tilt series, some of the bright particles contained dark centers, and when the tilt series is viewed as a movie, those particles appeared to ‘blink’ several times throughout the angular range. This behavior is attributed to the changing diffraction conditions of the crystalline gold particles as the sample is tilted. When the particle is in an orientation that produces strong diffraction, the increased inelastic signal of the gold is scattered outside the objective aperture by diffraction, i.e., mixed elastic–inelastic scattering occurs. Despite this fluctuating contrast effect, the IMOD program was able to track the positions of the fiducial markers throughout the double tilt series.

Representative projected phosphorus maps at 0°, 10°, 20°, 30°, 40° and 50° from the two-axis tilt series in Figs. 4a–f, respectively, indicate the presence of ribosomes situated in rows along the membranes of endoplasmic reticulum. From the C. elegans atlas [39], it appears that the region imaged is probably a hypodermal cell, which is one of the more common cell types in the organism. Even at a tilt angle of 50° in Fig. 4f, the phosphorus distribution is clearly evident demonstrating that the specimen is thin enough in the beam direction for the background shape to remain constant.

Slices of thickness 1.85 nm through a small region of the three-dimensional reconstructed volume are shown in Fig. 5a; for the purpose of illustration, only one half of the slices are presented in the figure, i.e. with 3.7 nm spacing. Thirteen well-defined features, situated at different heights within the approximately 60 nm thickness of the section, appear and then disappear through the stack of slices. These bright features with high phosphorus concentration have diameters in the range 15–20 nm. From their size and distribution within the section, the particles are identified as individual ribosomes [40]. It was possible to determine the relative phosphorus content of the different ribosomes in Fig. 5a by summing the phosphorus signal throughout the slices. The intensities were integrated in circular regions approximately 20 nm in diameter around the particles slice by slice. For each particle, the integrated intensities from the different slices were added to give the total phosphorus signal of...
Fig. 4. (a–f) Six images from the phosphorus tilt series (a) 0°, (b) 10°, (c) 20°, (d) 30°, (e) 40°, (f) 50°, obtained by ratio imaging. Even at a tilt of 50°, the phosphorus distribution is clearly visible. Bar=200 nm.

Fig. 5. (a) Slices through three-dimensional phosphorus reconstruction showing a small region of cell. Thirteen ribosomes are evident at various heights in the section. The slice thickness is 1.85 nm, and for clarity only every second slice is represented. Image width=100 nm. (b) Histogram of phosphorus content for the 13 ribosome particles in the slices through the reconstructed specimen volume shown in (a).
that ribosome. The results for the 13 ribosomes are presented as a frequency histogram in Fig. 5b. The relative standard deviation of the phosphorus signal for all 13 particles is 17% and nine of the centrally located particles gave a standard deviation of only 6%, suggesting that some of the ribosomes may have been cut in the sectioning process.

The tomographic phosphorus distribution is visualized three-dimensionally by the volume-rendered representation in Figs. 6a and b obtained using the Amira software. Vertical slices through the volume in the x- and y-directions (Fig. 6a) show the distributions of ribosomes within the section; more than one hundred ribosomes are evident within the rendered volume. The three-dimensional arrangement of ribosomes is more evident in Fig. 6b, which shows a small region of the tomographic reconstruction at higher magnification.

4. Discussion

We have shown that it is possible to obtain three-dimensional phosphorus distributions by tomographic reconstruction of energy-filtered electron micrographs. Next, we consider the sensitivity and spatial resolution of the technique. It is known that the eukaryotic ribosome contains approximately 7000 phosphorus atoms corresponding to the 7000 bases of ribosomal RNA (rRNA) [40]. In the reconstructed volume, each ribosome contains approximately 200 voxels, so on average there are about 35 phosphorus atoms per voxel in the ribosome. The atomic fraction of phosphorus in the projected volume encompassing a ribosome can be estimated from the density of the section. Assuming that the specimen has an average density of $1 \text{ g cm}^{-3}$ for the embedding resin and the biological components, a cylindrical volume of diameter 20 nm and a height equal to section thickness of about 60 nm, would contain a mass of about 11 MDa, including 3.5 MDa for the ribosome. The cylindrical volume of the specimen encompassing the ribosome would therefore contain approximately $9 \times 10^5$ carbon, nitrogen and oxygen atoms. Ignoring hydrogen, the atomic fraction of phosphorus in the volume of the ribosome would, therefore, be only 0.8 at.%, which corresponds to a dry weight concentration of approximately 700 mmoles/kg. Such phosphorus concentrations are typical of those imaged in thin sections by standard energy-filtered TEM.
Because of the large number of images recorded at different tilts, the minimum detectable number of atoms in a voxel of the 3-D reconstruction should be smaller than the minimum detectable number of atoms in a pixel in the projected 2-D map. This prediction relates to the principle of dose fractionation that has been discussed previously for conventional electron tomography [41].

In our data acquisition, the tilt series was constrained to $\pm 55^\circ$ due to the geometry of tilt holder, which obstructed the electron beam at higher tilt angles. However, even if a higher range of tilt angles were accessible, it would probably not have been possible to take advantage of tilt angles higher than $60^\circ$ for acquiring phosphorus tomograms at the beam energy of 120 keV used in this study. This is because plural inelastic scattering would eventually reduce visibility of the phosphorus L$_{2,3}$ edge and thus invalidate the background subtraction when the tilt angle was increased. The limited angular range of $\pm 55^\circ$ for the two orthogonal tilt axes corresponds to a ‘missing pyramid’ in the acquired tilt data. This is manifested by some distortion of the reconstructed features in the $z$-direction (Fig. 6). If the tilt series were acquired using 300 keV electrons instead of the 120 keV electrons used in the present study, and if a higher tilt holder were used, the quality of the reconstruction should be significantly improved.

Spatial resolution of elemental tomography is clearly limited by radiation damage. The dose that was used to record the two-axis image tilt series at the phosphorus L$_{2,3}$ edge was approximately $10^7$ e/\(\text{nm}^2\). Although this is a very high radiation exposure, we have demonstrated that phosphorus is retained in cellular structures such as ribosomes during acquisition. High-resolution information about the phosphorus distribution will undoubtedly be lost, and it remains to determine what spatial resolution is retained in the phosphorus tomogram. There is evidence that the plastic section partially collapses in the direction perpendicular to its plane. The observation that the phosphorus signal remains intact during tomographic acquisition implies that meaningful quantitative information about phosphorus concentration is obtainable in three dimensions even though some redistribution of the atoms will have occurred.

Finally, we consider how elemental tomography might be applied to biological systems. One application of particular interest involves determining the three-dimensional organization of nucleic acids and associated proteins in the cell nucleus. Conventional tomography of stained plastic sections has already been used to visualize splicosomes in isolated chromosomes [6]. Other work has made use of electron spectroscopic imaging to obtain two-dimensional phosphorus maps of structures within the cell nucleus such as centers of replication and transcription [17,18,42]. An advantage of phosphorus imaging relative to specific staining methods, is that phosphorus contrast is intrinsic. With staining techniques, there can be ambiguity about whether heavy atoms produce positive or negative staining or both. This ambiguity is avoided with phosphorus imaging, which shows directly the locations of nucleic acid. Furthermore, by combining phosphorus tomography with nitrogen tomography, it might be possible to distinguish between nucleic acid, which contributes both phosphorus and nitrogen signals, and protein, which only contributes a nitrogen signal. Such an approach has already been developed in two-dimensional electron spectroscopic imaging of the nucleus [42]. By extension, electron spectroscopic tomography might prove to be a promising technique for investigating the structure of the cell nucleus in three dimensions.

5. Conclusions

We have shown that energy-filtered electron tomography can be applied to unstained biological specimens to provide three-dimensional elemental distributions. For sufficiently thin plastic sections of freeze-substituted, unstained cells, ratio imaging at energy losses above and below the phosphorus L$_{2,3}$ edge yields valid maps of the projected phosphorus distribution at beam energies of 120 keV. The projected phosphorus maps can be aligned using colloidal gold particles deposited on the surface of the section, which are recognizable
in the pre-edge images. Three-dimensional volume rendering of the phosphorus distribution clearly revealed the ribosomal RNA localized within regions of diameter 15–20 nm, as well as the three-dimensional distribution of the ribosome particles within the context of the cell. The sensitivity of the technique can be assessed from the known total phosphorus content of approximately 7000 atoms in each ribosome. Bright features in the three-dimensional reconstruction contained about 35 phosphorus atoms per voxel. Although a high electron dose is required to record two-axis tilt series, specimens are sufficiently stable to allow image alignment and tomographic reconstruction.

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