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Three-dimensional elemental mapping of phosphorus by quantitative electron spectroscopic tomography (QuEST)

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Abstract

We describe the development of quantitative electron spectroscopic tomography (QuEST), which provides three-dimensional distributions of elements on a nanometer scale. Specifically, it is shown that QuEST can be applied to map the distribution of phosphorus in unstained sections of embedded cells. A series of 2D elemental maps is derived from images recorded in the energy filtering transmission electron microscope for a range of specimen tilt angles. A quantitative 3-D elemental distribution is then reconstructed from the elemental tilt series. To obtain accurate quantitative elemental distributions it is necessary to correct for plural inelastic scattering at the phosphorus L_{2,3} edge, which is achieved by acquiring unfiltered and zero-loss images at each tilt angle. The data are acquired automatically using a cross correlation technique to correct for specimen drift and focus change between successive tilt angles. An algorithm based on the simultaneous iterative reconstruction technique (SIRT) is implemented to obtain quantitative information about the number of phosphorus atoms associated with each voxel in the reconstructed volume. We assess the accuracy of QuEST by determining the phosphorus content of ribosomes in a eukaryotic cell, and then apply it to estimate the density of nucleic acid in chromatin of the cell's nucleus. From our experimental data, we estimate that the sensitivity for detecting phosphorus is 20 atoms in a 2.7 nm-sized voxel.

Keywords

Electron tomography; energy-filtered transmission electron microscopy; EFTEM; electron energy loss spectroscopy; phosphorus; ribosome; nucleus; chromatin; 3-D reconstruction; quantitative elemental imaging

1. Introduction

It is now possible to map reliably in two dimensions the nanoscale distributions of specific elements by using the technique of energy filtered transmission electron microscopy (EFTEM). Elemental mapping is achieved by collecting images of inelastically scattered electrons that have excited core level electrons of specific atoms in suitably thin specimens. EFTEM mapping has been applied successfully to biological systems as well as to a wide range of materials

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(Krivanek, et al., 1995; Egerton, 2003; Hofer and Warbichler, 2004). In biological applications, elemental maps of unstained sections provide information about the 2-D distributions of various classes of biomolecules within a sectioned cell (König, et al, 2007). For example, the nitrogen signal provides information about the total distribution of biomolecules, including proteins and nucleic acids (Goping, 2003; Bazett-Jones, et al., 1999). Sulfur indicates the presence of proteins that contains high levels of the amino acids, cysteine and methionine (Leapman et al., 1997). Phosphorus reveals the distribution of nucleic acids, phosphorylated proteins and phospholipids (Korn, et al., 1983; Ottensmeyer, 1984; Bazett-Jones, et al., 1988), since small molecules containing phosphorus are mostly removed in plastic-embedded preparations. If the relative concentrations of phospholipids and phosphoproteins are low enough, the phosphorus distribution provides information about the packing density of DNA within the chromatin of a cell's nucleus (Bazett-Jones, et al., 1996; Bazett-Jones and Hendzel, 1999; Bazett-Jones, et al., 1999). However, a quantitative interpretation of elemental distributions requires a 3-D analysis due to ambiguity caused by overlapping structures in 2-D projections.

Electron tomography (ET) has become a well-established technique for 3-D structural analysis at the subcellular level (Frank, 1992; Frank, 2006; McIntosh, et al., 2005; Koster, et al., 1997). In ET, a series of images is acquired as the specimen is tilted through a range of angles, from which the 3-D electron density distribution of the specimen is derived. By combining ET with EFTEM, we have already shown qualitatively that it is possible to reconstruct the 3-D distribution of specific chemical elements within a cellular volume (Midgley and Weyland, 2003; Leapman, et al., 2004). Here we introduce *quantitative electron spectroscopic tomography* (QuEST), and show how it can be used to determine 3-D distributions of phosphorus and hence nucleic acid within subcellular volumes. Determination of quantitative 3-D elemental distributions using QuEST depends only on a single parameter that is not instrument dependent, namely the inelastic scattering cross section for core electron excitation.

Understanding EFTEM imaging requires knowledge of the electron energy loss spectral shape at energies close to the excitation edge of a particular element. A schematic diagram of the spectrum near the phosphorus $L_{2,3}$ edge, corresponding to excitation of $2p$ electrons is shown in Fig. 1a. Regions of integrated pre-edge intensity at 120 ± 10 eV and post-edge intensity at 152 ± 10 eV are indicated, from which the phosphorus signal can be calculated at each projection in the tilt series. In Fig. 1b, we outline the three main steps in the generation of a 3-D elemental distribution, i.e., the initial acquisition of raw energy-selected images, the background subtraction and calculation of 2-D net phosphorus distributions, and the reconstruction procedure.

Although phosphorus is a major constituent of nucleic acids, the phosphorus $L_{2,3}$ signal obtained from EFTEM images of sectioned plastic-embedded cells is relatively weak. Whereas about 5% of the non-hydrogen atoms in DNA are phosphorus, the atomic fraction of phosphorus in a region of plastic-embedded chromatin within a cell nucleus typically constitutes only one percent of the total number of carbon, nitrogen and oxygen atoms. The dilution of phosphorus by proteins associated with the DNA and by the surrounding embedding medium results in the phosphorus signal always being a small fraction of the background intensity. Therefore, special attention must be given to subtracting the core edge signal from the background in the energy loss spectrum (Egerton, 1996; Haking, et al., 1999). Another challenge in attempting to map elements such as phosphorus in 3-D is the increased effective specimen thickness at high tilt angles, which can further limit the reliability of the background subtraction, particularly for small signal to background ratios (Leapman and Rizzo, 1999). Furthermore, biological specimens are susceptible to beam damage (Leapman, 1986) and previously it has not been shown that an entire image tilt series can be acquired before the sample changes shape. If deformation occurs, automated specimen drift correction by image

cross correlation during acquisition of the tilt series or the subsequent precise alignment of the images after acquisition can be compromised. Finally, as in conventional ET, the fiducial markers used for image alignment must be observable in the energy-filtered images (Mastronarde, 1997).

2. Experimental Methods

2.1 Specimen preparation

To obtain the unstained sections for elemental mapping in EFTEM, *Drosophila* larvae were collected in 15% sucrose and transferred into sample carriers. The samples were frozen in an HPM10 high-pressure freezing machine (Bal-Tec, Balzers, Switzerland). The frozen larvae were freeze-substituted in acetone containing 0.2% glutaraldehyde (Ted Pella, Redding, CA) at -90°C for 3 days and then slowly warmed (5 C/h) to 20°C using an EM-AFS freeze-substitution system (Leica Microsystems, Wetzlar, Germany). After rinsing several times in acetone, the samples were infiltrated with Epon-Aradite (Ted Pella, Redding, CA) and polymerized at 60°C for 2 days. The sections were cut on a Reichert Ultracut E (Leica Microsystems, Wetzlar, Germany) to a thickness of 90–150 nm, and picked up on 400 mesh bare copper finder grids (EM Sciences, Hatfield, PA). To ensure the stability of the sections under the electron beam, 5–10 nm carbon films were evaporated directly onto the copper grids containing the specimens using a Auto 306 evaporator (BOC Edwards, West Sussex, UK). Gold particles were used as fiducials for tracking during acquisition of the tilt series and alignments for tomographic reconstruction. The grids were glow-discharged in air for 30 sec and 10 nm colloidal gold (SPI Inc., West Chester, PA) was deposited on top and bottom of the grids. After 5 minutes, the samples were thoroughly washed with de-ionized water to remove excess reagents.

2.2 EFTEM tomography

The tomographic tilt series were obtained using a Tecnai TF30 transmission electron microscope (FEI Inc., Eindhoven, Netherlands) operating at a beam voltage of 300 kV and equipped with a Tridiem post-column imaging filter (Gatan Inc., Pleasanton, CA). To optimize contrast in the unstained plastic embedded sections a 40- μm diameter objective aperture was inserted. The acquired images of the 2048×2048 pixel Ultrascan cooled CCD detector were hardware-binned to 512×512 pixels to improve the signal-to-noise ratio, resulting in a final pixel size of 2.7 nm. The tomography sample holder (Fischione Instruments, Inc., Export, PA) allowed a maximum tilt range of $\pm 80^{\circ}$. However, we have generally restricted the tilt range to $\pm 69^{\circ}$, since outside this range plural inelastic scattering increases precipitously and causes inaccuracy in the elemental distributions. To limit the total electron dose on the specimen, a tilt increment of 3° was selected, which resulted in 47 images in each of the four series (i.e., a total of 188 images). To acquire an orthogonal tilt series for dual-axis reconstruction, the grid was rotated in plane by 90° and the same number of 188 images acquired. The EFTEM tomography software (Gatan, Inc., Pleasanton, CA) (Harmon, et al., 2005), written in Digital Micrograph provided automated acquisition of data by controlling the spectrum energy offset and the width of the energy-selecting slit. In addition, the acquisition software controlled the tilt of the specimen goniometer, the excitation of the image deflection coils, and objective lens, just as in standard electron tomography. A high-speed shutter allowed us to acquire successively four images (zero-loss, unfiltered, pre-edge and post-edge) at each tilt angle without saturating the CCD detector while maintaining the same illumination on the specimen. Cross correlation of zero-loss images enabled correction for specimen drift, as well as correction for focus change using the image shift, induced by tilting the beam. Zero-loss image series were acquired using a 20-eV slit width. Energy-selected series were obtained in the vicinity of the $\text{P L}_{2,3}$ edge using a 20-eV slit width with a pre-edge window at 120 eV and a post-edge window at 152 eV (Fig. 1). The readout time for each energy-selected image was 4

s and each image was summed in 4 readouts. For all the data acquisition, it was critical for the dark current to be subtracted properly, for the CCD gain to be normalized and for the image filter to be tuned to provide an isochromaticity of less than 1 eV across the full image field. A simplified flow diagram indicating the sequence of the acquisition process is shown in Fig. 2.

3. Determination of 2-D phosphorus distributions

Quantitative elemental mapping in 2-D requires the acquisition of four separate images: a phosphorus post-edge image with pixel intensity I^{post} ; a pre-edge image with pixel intensity I^{pre} ; a zero-loss image with pixel intensity I^{zero} ; and an unfiltered image with pixel intensity I^{unfilt} . Taken together the zero-loss and unfiltered images provide a measure of the specimen thickness, which is used to correct for plural inelastic scattering.

To calculate the intensities in absolute number of atoms, we adopt a modified two-window approach (Aronova, et al., 2007). Provided the background intensity remains constant over the field of interest, the simplest expression for the net core-edge signal in 2-D is

$$S(\Delta, \beta) = I^{post} - kI^{pre}, \quad (1)$$

where Δ is the energy width of the integration window above the core edge and β is the collection semi-angle of the scattered electrons defined by the objective aperture (Egerton, 1979). Here k is the ratio of post-edge to pre-edge image intensities for a specimen that does not contain phosphorus, and it is assumed that k remains constant across the image field, which is valid if the specimen has a uniform thickness. As the specimen is tilted during the acquisition of the tomographic series, the electron path length within the specimen increases, doubling at 60° . It therefore becomes evident that k does not remain constant throughout the tilt series. Furthermore, for a given tilt angle, variations in mass per unit area from pixel to pixel can result in significant changes in k . Using a constant k -value will result in an erroneous phosphorus signal. Thus to obtain an accurate and reliable 3-D phosphorus distribution, the thickness dependent k -ratio is required (Aronova, et al., 2007).

The specimen thickness can be conveniently expressed in units of total mean free path λ for inelastic scattering, which is determined from the fraction of transmitted electrons that are present in the zero-loss intensity (Egerton, 1996) as

$$\frac{t}{\lambda} = \ln \left(\frac{I^{unfilt}}{I^{zero}} \right). \quad (2)$$

As the specimen is tilted and t/λ increases, plural inelastic scattering will affect the shape of the EELS background at each pixel. We can model these subtle changes in the spectrum and formulate a dependence of k on t/λ . For each tilt angle θ , it is possible to compute a k ratio map that can be used to determine accurately the phosphorus signal S_θ . Eq. (1) can now be rewritten as:

$$S_\theta(\Delta, \beta) = I^{post} - k_\theta(t/\lambda)I^{pre}. \quad (3)$$

From the signal S_θ at tilt angle θ , we obtain a projected 2-D phosphorus distribution corresponding to the number of phosphorus atoms associated with each pixel according to the following formulation:

$$n_\theta = \frac{S_\theta(\Delta, \beta)}{I^{zero}(\Delta, \beta)\sigma(\Delta, \beta)} \quad (4)$$

Here, $\sigma(\Delta, \beta)$ is the phosphorus inelastic scattering cross-section per atom for an energy window above the edge of width Δ and for a collection semi-angle β . $\sigma(\Delta, \beta)$ is given in units of square

nanometers per atom. As discussed previously, the statistical error in the number of phosphorus atoms per pixel in a given projection is given by (Aronova, et al., 2007):

$$\delta n_{stat-2D} = \frac{\sqrt{k(1+k)I_{\theta}^{pre}}}{I_{\theta}^{zero} \sigma(\Delta, \beta)} \times s^2 \quad (5)$$

where s is the size of a pixel in units of nanometers.

4. Determination of 3-D phosphorus distributions

The elemental distribution within the specimen volume is computed from the measured 2-D projections by means of a 3-D reconstruction algorithm. Using a ray sum (Goitein, 1972), we can express the 2-D number density of phosphorus atoms $n_{\theta}(x, y)$ projected onto a plane perpendicular to the beam direction having pixel coordinates x, y , in terms of a 3-D number density of phosphorus atoms within the specimen volume $v(x', y', z')$ having voxel coordinates x', y', z' . For example, as shown in Fig. 3a for 0° specimen tilt angle, $n_{\theta}(x, y)$ can be expressed in terms of $v(x', y', z')$ as

$$\sum_{x', y', z'} v(x', y', z') \delta_{x, x'} \delta_{y, y'} = n_{\theta=0}(x, y), \quad (6a)$$

where $\delta_{x, x'}$ is a Kronecker delta function, which has a value of one, when x equals x' and is zero otherwise. As the specimen is tilted, the summation of the voxel values becomes more complicated, since the rays no longer pass through all the voxels equally. Figure 3b shows the x - z cross section with rays passing through a fraction of the voxel, labeled as area ABC.

Therefore, derivation of an expression equivalent to Eq. (6a) for projections $n_{\theta}(x, y)$ at nonzero values of θ requires a nonuniform weighting factor w . Here w corresponds to a fraction of the voxel area that is intersected by the two adjacent rays; in this case, it is simply a ratio of the area ABC to the pixel area, labeled as α^2 . Hence, the ray sum for any tilted angle θ can be written as:

$$\sum_{x', y', z'} v(x', y', z') w_{\theta}(x', y', z'; x, y) = n_{\theta}(x, y), \quad (6b)$$

where w_{θ} now depends on the coordinates of the voxels and pixels, and the tilt angle θ . For a tilt angle of $\theta = 0$, the weighting factor $w_{\theta=0}(x', y', z'; x, y)$ reduces to $\delta_{x, x'} \delta_{y, y'}$ as given in Eq. (6a).

Although an exact treatment of errors in the 3-D volume is complicated, it is possible to estimate the statistical error in the number of phosphorus atoms per voxel by considering that the 2-D error given by Eq. 5 must originate from the combined errors from each slice of the reconstructed tomogram. If there are N slices and the errors in each slice are uncorrelated, then the contribution of the errors from each slice adds in quadrature to give a 2-D error that is $\sqrt{N}$ larger than the error in each slice. The 3-D error is then given by:

$$\delta n_{stat-3D} = \frac{\delta n_{stat-2D}}{\sqrt{N}}, \quad (8)$$

This simple analysis provides a useful estimate of the minimum noise level imposed by counting statistics but it ignores errors introduced by the reconstruction algorithm and correlated noise between successive slices in the reconstruction. The theoretical statistical error in the number of phosphorus atoms per voxel is plotted logarithmically as a function of t/λ for different numbers of incident electrons/nm² (Fig. 4). Typically, we acquire tilt series of specimens with a relative thickness of 0.3 to 0.5 inelastic mean free paths with a total exposure of 10^7 electrons/nm², from which we predict a theoretical minimum statistical error of between 9 and 15 P atoms/voxel.

Although a number of different algorithms exist for 3-D tomographic reconstruction (Kak and Slaney, 1988; Herman, 1980; Gordon, et al., 1970; Andersen and Kak, 1984), we choose the simultaneous iterative reconstruction technique (SIRT) (Gilbert, 1972a) to compute quantitative 3-D elemental distributions from our EFTEM images. The SIRT approach makes use of a back projection algorithm, as does the commonly used Fourier weighted back projection (WBP) method (Gilbert, 1972b). However, in the SIRT approach, a solution for the reconstructed 3-D object is derived from a set of linear equations via a ray sum in real space (Fig. 3), whereas in the WBP technique the back projection is done using Radon or Fourier transforms (Shepp, 1980; Deans, 1983). In performing WBP, the original projections are mapped in frequency space by using the central slice theorem (De Rosier and Klug, 1968), from which it is evident that Fourier components at lower spatial frequency are oversampled relative to Fourier components at high spatial frequency. Furthermore, when reverting to real space in the WBP method to obtain the 3-D solution, the Fourier components at higher spatial frequencies must be interpolated to fill gaps in the missing data. To avoid artifacts in the solution of the 3-D structure with the WBP approach, it is common to employ filters. The use of R-filtering, for example, diminishes the importance of the lower frequencies. However, filtering can generate artifacts and, in the case of R-filtering, the low spatial frequency Fourier components of reconstructed volume will have arbitrary offsets. In addition, ringing noise will be generated at higher frequencies in the real space reconstruction. Thus, although the WBP approach is quick and useful for 3-D structure determination, it is not well suited for quantitative 3-D elemental mapping because the original intensities are not preserved (Friedrich, et al., 2005).

The SIRT algorithm does not use filtering when the 3-D solution is generated via back projection. Instead, the 3-D solution is refined by successive iterations, in which the differences between the solution and the original projections are minimized. By simulating elemental tomographic data, we have found that the optimal number of iterations depends on (i) the amount of noise in the data, (ii) the number of original projections, and (iii) the tilt angle increment. In addition, the size of the features of interest and the intensity distribution within the volume are important parameters for determining the optimal number of iterations. To assess the convergence of the algorithm, we compute two functions: the discrepancy γ and the coefficient of determination (COD), both of which have been used in previous work (Gilbert P., 1972; Draper and Smith, 1981). These functions are defined as follows:

$$\gamma = \left[\frac{\sum_i (n_i - n_i^q)^2}{\sum_i (n_i - n_i^0)^2} \right]^{1/2} \quad (7)$$

$$COD = \frac{\sum_i \left((n_i - \bar{n})(n_i^q - \bar{n}^q) \right)}{\sqrt{\sum_i (n_i - \bar{n})^2 \sum_i (n_i^q - \bar{n}^q)^2}}$$

In these expressions, n_i is the voxel value of the simulated volume, n_i^0 and n_i^q the voxel values in the reconstructed volume after the 0th and qth iterations, respectively, and $\bar{n}$ and $\bar{n}^q$ are the mean voxel values in the simulated and reconstructed volumes, respectively. The behavior of γ provides a way of assessing the convergence of the data as a desirable solution is approached. The behavior of the COD indicates the degree of correlation between the reconstructed data and the known simulated volume. The true 3-D solution occurs when the COD approaches unity, and when γ approaches zero.

Simulated randomly distributed ribosomes are used to demonstrate the dependence of the convergence of the solution as a function of the number of iterations for various noise levels,

tilt ranges, and tilt increments. Fig. 5a and 5b indicate the behavior of γ and COD, respectively, as a function of iteration number for various noise levels: 0, 10, 20, 30 and 40 P atoms per pixel in the zero tilt projection. From these curves, it is evident that the solution becomes more and more inaccurate as the number of iterations increases, and that the optimal number of iterations decreases as the noise in the data increases. As the number of iterations is increased beyond the optimal number, the solution differs from the simulated object, since the noise in the reconstructed volume is forced to match to the original noise in the projection. From Fig. 5c, where the tilt range is shown as a function of number of iterations, it is evident that the solution approaches the simulated object when the tilt range is the largest, which is expected based on the additional information that is utilized in the reconstruction. For similar reasons, it is evident from Fig. 5d that the data collected with a smaller tilt interval yields a solution that is closer to the simulated object as the number of iterations is increased, although the advantage of increasing the number of tilts is less important than increasing the tilt range.

Similar trends for γ and the COD have been published for other types of data (Gilbert P, 1972; Tong, et al., 2006). Considering all these factors, we typically run the SIRT for 20-30 iterations to reconstruct our 3-D phosphorus distributions, for which the noise level at a zero tilt projection is approximately ± 20 P atoms per pixel, with data recorded over a $\pm 69^\circ$ tilt range and a 3° tilt increment. The experimental data discussed in this paper is closest to simulation curve labeled with triangles in Fig. 5b

The positivity of the solution is not reinforced in our algorithm, since we want to allow for negative numbers of atoms in the reconstructed volume to enable a proper treatment of statistical fluctuations. For this reason, an additive SIRT rather than a multiplicative SIRT was used (Gilbert P., 1972). As shown in Fig. 5c, with a limited number of acquired projections, artifacts are produced due to loss of information about the object (i.e., missing wedge effect). Such artifacts are easily visible when single axis tomographic series are acquired. By acquiring two tomographic tilt series about two orthogonal axes, and combining them in Fourier space, the missing wedge becomes a missing pyramid and artifacts are substantially reduced as demonstrated in previous tomographic reconstructions with the WBP and SIRT algorithms (Midgley and Weyland, 2003; Penczek, et al., 1995).

Our additive SIRT algorithm, written in MATLAB and FORTRAN, was used to reconstruct single-axis and dual-axis tilt series. Dual-axis tomograms were combined into one tomogram in Fourier space by using the IMOD software (Kremer, et al., 1996). The resultant tomograms were then volume-rendered using the Amira software package (Mercury Sys. 2006).

Using the same simulated data that allowed us to understand the dependence of the quality of reconstructed data on several parameters as a function of number of iterations (Figs. 5a-d); we have investigated the error in the reconstructed volume relative to the statistical error due to Poisson counting statistics in the initial simulated projections. For this, we compare the standard deviation σ of the z-projected reconstructed volume of 30 slices with the σ of the 0° tilt simulated projection as a function of number of iterations. This relation is evident in Fig. 6, which shows that $\sigma_{reconstructed}$ approaches $\sigma_{simulated}$, as number of iterations increases. However, it is clear from Fig. 5 that increasing the number of iterations beyond an optimal number introduces errors when Poisson noise is present. Therefore, for a particular thickness, and a given number of iterations, one can calculate $\sigma_{reconstructed}$ and divide it by the ratio obtained from the curve. For example in using 30 iterations to obtain a σ of 6 P atoms in the reconstructed volume, the actual σ that should be reported is $6/0.31$ or ~ 19 P atoms. This enables us to obtain a better estimate of sensitivity for the detection of phosphorus atoms from our experimental data.

4. Results and discussion

Using QuEST, we generate 3-D phosphorus maps and investigate the number of phosphorus atoms associated with ribosomes and with regions of chromatin, from which DNA density can be determined. We also estimate errors in the 3-D elemental distributions and discuss some potential applications of QuEST.

First, it is necessary to consider the effect of electron irradiation on the quantification of the elemental maps. It is well known that high-energy electrons damage biological specimens and can induce shrinkage of irradiated specimens in the electron microscope, and the shrinkage of sectioned plastic-embedded cells is largest in the z direction, i.e., perpendicular to the plane of the section (Braunfeld, et al., 1993; Egerton, et al., 2006). We have determined the magnitude of the shrinkage factor for our experimental conditions by acquiring tomograms from sectioned Epon embedding material, onto whose surfaces 10 nm-diameter colloidal gold particles had been deposited. The thickness of a region of the specimen was first determined from a tomogram acquired using scanning transmission electron microscopy (STEM) with a total dose of $2 \times 10^3 \text{ e}^-/\text{nm}^2$ as described by Sousa et al. (Sousa, et al., 2007). Images in the specimen y - z plane obtained by projecting through 90 to 100 slices in the x -direction are presented in the right column of Fig. 7. For speed of computation, a simple WBP reconstruction algorithm was used to reconstruct the STEM tomogram, in which it can be seen that the gold fiducials visible on the surfaces of the plastic section are elongated in the z direction. The thickness was measured again by STEM tomography in the same region of the specimen after exposing the specimen to a high total dose of $5 \times 10^7 \text{ e}^-/\text{nm}^2$, necessary to acquire energy filtered images. Images in the y - z plane of the reconstructed volume after exposure of the specimen to the high electron dose are presented in the right column of Fig. 7. After performing the calculations from the three regions shown in Fig. 7, we find that electron irradiation results in a lateral shrinkage factor $\xi_z = 0.55$ in the z direction, and lateral shrinkage factors $\xi_x = \xi_y = 0.88$ in the x and y directions. Multiplication of the lateral shrinkage factors allows us to estimate the volume shrinkage factor,

$$\xi_V = \xi_x \times \xi_y \times \xi_z, \quad (9)$$

from which we find that the volume shrinkage ξ_V is 0.43. This large reduction in volume must be taken into account in interpreting measured densities of phosphorus-containing structures such as chromatin in the cell nucleus, as we shall discuss later.

In our measurements, zero-loss and unfiltered tomographic series are used to generate the t/λ -tilt series required to correct for plural scattering at each tilt angle. This enables us to generate a thickness-dependent k -map tilt series, which is multiplied by the pre-edge tilt series, and then subtracted from the post-edge tilt series (Aronova, et al., 2007). Then we make use of Eq. 4 to compute a tilt series whose pixels values correspond to the number of atoms of phosphorus per pixel projected along the beam direction. The alignment obtained from the zero-loss tilt series, using the gold particles as fiducials, was then applied to this tilt series using the IMOD software. At this stage, the aligned tilt series is ready for reconstruction to obtain the 3-D phosphorus distribution.

To demonstrate the derivation of the 3-D phosphorus distribution from unstained cells, we have recorded EFTEM tomographic data from *Drosophila* larvae. Examples of raw images recorded at 0° tilt angle are shown in Fig 8, where (a) is an unfiltered image, (b) is zero-loss image, and (c) and (d) are energy-selected images recorded at energy losses of 120 eV and 152 eV, respectively, corresponding to pre-edge and post-edge windows for the P $L_{2,3}$ excitation. By processing these four images according to Eqs. 2 and 3 and by incorporating values for the phosphorus inelastic cross section $\sigma(A, \beta)$, we use Eq. 4 to calculate the 2-D phosphorus distribution, which is shown in Fig. 8e for a specimen tilt of 0° . Tightly packed heterochromatin

is clearly visible in the lower right region of the image containing the nucleus (nuc) whose boundary is indicated by a dashed line, and a high concentration of ribosomes is evident in the cytoplasm (cyt) situated in the lower left. From such 2-D images, it is possible to estimate the number of phosphorus atoms in structures of interest like ribosomes (Abolhassani-Dadrasa, et al, 1998; Ottensmeyer and Andrew, 1980). However, 2-D analyses usually lead to overestimation of the number of atoms due to phosphorus contributions from overlapping structures.

To reconstruct 3-D distributions of phosphorus we use 30 iterations of the additive SIRT algorithm for two orthogonal tilt series, so that the value at each voxel is equal to the number of phosphorus atoms associated with that voxel. Fig. 9a is the volume rendered 3-D phosphorus distribution of the same cell shown in Fig. 8, viewed down the z-axis, i.e., through the specimen thickness. The color-coding of the phosphorus distribution in Fig. 9a distinguishes between the different cellular regions that are analyzed, i.e., the cytoplasm containing ribosomes (blue-green) shown at higher magnification in Fig. 9b, and the nucleus (red-orange) shown at higher magnification in Fig. 9c. The tomographic reconstruction of another cell shown in Figs. 9d, 9e and 9f indicates a similar distribution of phosphorus in regions of cytoplasm containing ribosomes and in regions of the nucleus containing heterochromatin. It is evident that the gold markers deposited on the specimen surfaces to facilitate tracking during data acquisition and subsequent fine alignment of the tilt series, are the brightest red/orange particles shown in the reconstructed volume. The gold fiducials are visible in the phosphorus map because the gold spectrum has a nonzero intensity in the region of the phosphorus $L_{2,3}$ edge, as shown by the measured energy loss spectrum from a gold film in Fig. 10. The regions containing ribosomes and chromatin in Fig. 9a were selected for detailed analysis of the phosphorus content. A region of plastic embedding was used to estimate the experimental statistical error in the numbers of phosphorus atoms, which is essential in establishing the validity of the QuEST technique.

To obtain the distribution of the number of phosphorus atoms contained in ribosomes, we first select a 3-D volume that is likely to be a ribosome and sum the voxel values inside this volume. A MATLAB program was written to perform this calculation automatically, as follows. We first distinguish the ribosomes from the cell background by removing from the 3-D tomogram the voxels that have intensities lower than a threshold of 40 phosphorus atoms. After thresholding, a cluster algorithm is adopted to group the voxels into clusters as follows. (1) Since the diameter of a ribosome is taken to be 7 voxels $\times$ 2.7 nm, a sphere of radius of 3.5 voxels is drawn around every voxel. For example, if there are N voxels remaining after thresholding, we draw N overlapping spheres. (2) For each sphere, the voxel intensities are summed. (3) Among all the spheres, the sphere of the highest integrated intensity is chosen and the voxels inside the sphere are designated to a cluster. (4) We then remove these voxels and return to step (1) until every voxel is removed. Once the voxels are clustered, we only designate clusters of 80 voxels or more as putative ribosomes, since the diameter of a ribosome is known to be $\sim$ 20 nm and therefore that it contains approximately 155 voxels in total. Clusters containing fewer than 30 voxels are attached to larger nearby clusters that have the maximum number of neighbors.

A histogram of the phosphorus content of the ribosomes, as a well as a histogram of their volume, and a histogram of the phosphorus content of a region of plastic embedding material are plotted in Fig. 11a. We find that in a volume of 152 ± 28 voxels there are 7900 ± 1900 P atoms, which agrees with the known phosphorus content of about 7000 P atoms and is also consistent with the known ribosome volume of $\sim$ 212 voxels. Although the measured volume of the ribosome is smaller than its known size presumably due to radiation induced shrinkage, the ribosome does maintain its “spherical” shape in the SIRT reconstruction, as demonstrated for the fiducial markers in Fig. 9g. Due to the electron beam exposure, the shrinkage of the ribosome volume is relatively small and is isotropic, and no other distortions are observed. The

measured phosphorus content is higher than expected, which might be explained by the presence of other phosphorus-containing molecules like phospholipids that become attached to the ribosomes via diffusion during the sample preparation. In this regard, it is known that the high pressure freezing method can destroy bilipid membrane, allowing the lipids to dissolve into the cell (Semmler, et al., 1998). In the plastic region, the phosphorous content found in a $7 \times 7 \times 7$ voxel cube, which is similar to a volume that a ribosome would occupy if it were placed in a cube, was -100 ± 300 P atoms ($\pm$ st. dev). Figure 11b shows distribution of phosphorus in a region of chromatin contained in a volume of $7 \times 7 \times 7$ voxels, with and without inclusion of the shrinkage factor of the section under electron irradiation. Since the chromatin is uniformly distributed inside the cell nucleus, the shrinkage of the Epon embedding material should be considered, when calculating the DNA density. Taking into account the volume shrinkage factor of 0.43, we obtain 5800 ± 600 P atoms in a volume containing $7 \times 7 \times 7$ voxels of chromatin. This corresponds to 0.5 to 0.9 phosphorus atoms per nm^3 , which is consistent with a model of 30 nm chromatin fibers that are tightly packed (Bazett-Jones and Hendzel, 1999;Schalch, et al., 2005). A summary of these results is found in Table 1.

To evaluate the sensitivity of the QuEST technique we compare the measured statistical errors in 2-D and 3-D data with theoretical values. Figure 11c shows a histogram of the number of phosphorus atoms per voxel from specimen regions containing plastic and regions containing chromatin. From these histograms we determine that there are 35 ± 37 phosphorus atoms in a 1 voxel-volume of chromatin and -1 ± 20 P atoms in a 1 voxel-volume of plastic. We have divided the measured standard deviation by the factor of 0.3, as given in Fig. 6, since the measured standard deviation of the reconstructed image is smaller than that allowed by statistical considerations. It is clear that we can distinguish chromatin from plastic by examination of a single voxel. Table 2 shows a summary of the calculated and predicted statistical errors for 2-D and 3-D data. From Fig. 4 the theoretical 3-D statistical error for the dose of $5 \times 10^7 \text{ e}^-/\text{nm}^2$ that we have used to obtain the energy filtered tomographic series is 6 P atoms, whereas from the experiment we find that it is 20 P atoms. The discrepancy here is due to other sources of error, as we have previously mentioned, which are related to data acquisition or processing. Nevertheless, overall agreement is still satisfactory.

Conclusions

We have shown that it is possible to use the QuEST technique to explore interesting properties of the cell nucleus such as the density of chromatin and the 3-D distribution of nucleic acid, by automated acquisition of zero-loss, unfiltered, $\text{P L}_{2,3}$ pre-edge and $\text{P L}_{2,3}$ post-edge images. With the help of an appropriate reconstruction algorithm, it is feasible to interrogate a voxel in the reconstructed volume and to extract the number of P atoms in an unstained plastic section of thickness 90-150 nm with a sensitivity of 20 P atoms per 2.7 nm-sized voxel.

Although this paper has primarily focused on phosphorus mapping, QuEST is a versatile technique that can be applied to map 3-D distributions of other biological elements such as sulfur, nitrogen, calcium and iron. For each of these elements it would be necessary to select optimal energies for pre-edge and post-edge windows, to calculate how plural inelastic scattering changes the k-ratio as function of specimen thickness, and to incorporate the corresponding partial cross sections for the core shell excitation. An application where imaging of more than one element might be valuable is mapping nitrogen together with phosphorus to separate in 3-D nucleic acid from other structures within the nucleus that are mainly composed of protein, a technique that has already been demonstrated in 2-D (Bazett-Jones, et al., 1999).

It must be kept in mind that application of QuEST is sensitive to many parameters, including sample preparation, data acquisition, and data processing, all of which can result in sources of error. To assess the sensitivity and accuracy of QuEST, it would be useful to develop a standard

biological structure containing a known numbers of phosphorus atoms to facilitate an independent calibration of the experimental data. Such standards are already employed in other types of measurements, for example in mass mapping of proteins using tobacco mosaic virus as an internal reference (Muller, et al., 1992). We are in the process of designing a standard for use in phosphorus mapping.

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Figure 1.

(a) Schematic representation of the electron energy loss spectrum near the $P L_{2,3}$ edge, indicating the pre-edge energy window at 120 ± 10 eV and the post-edge at 152 ± 10 eV.

(b) Simplified diagram showing determination of a 2-D phosphorus map by subtracting a suitably scaled pre-edge image from a post-edge image, and then the determination of a 3-D phosphorus map from a tomographic reconstruction of a 2-D phosphorus tilt series. Two features are shown in the pre-edge and post-edge images but only the one at the top right contains phosphorus.

Figure 2.

A flow diagram of EFTEM tomographic acquisition in which energy filtered pre-edge and post-edge are acquired, for a series of specimen tilt angles. Zero-loss and unfiltered images are also acquired to facilitate tracking for specimen drift and correction for plural scattering effects.

Figure 3.

Representation of the back projection technique, in which the 2-D projections are obtained from the 3-D densities $v(x'y'z')$ by ray summations. (a) To obtain the 0° tilt projection is simple since the x, y coordinates in 3-D and 2-D are the same. (b) To obtain 2-D projections for nonzero tilt angles requires modification of the ray summation by a weighting factor w because the summation rays intersect only a fraction of a 3-D voxel, such as the triangular area labeled ABC in the figure.

Figure 4.

Calculated statistical error ($\pm$ standard deviation) in number of phosphorus atoms per voxel as a function of t/λ , for a carbon matrix of thickness 80 nm. The errors are plotted on a logarithmic scale for four different incident electron doses of 10^5 , 10^6 , 10^7 , and $10^8 \text{ e}^-/\text{nm}^2$ for an accelerating voltage of 300 kV.

Figure 5.

Quality of the SIRT reconstruction from simulated 3-D phosphorus distributions, as a function of the number of iterations. (a) The discrepancy γ continues to decrease as the number of iterations increases if the raw data does not contain Poisson noise; however, in the presence of noise, indicated by an equivalent standard deviation in numbers of phosphorus atoms (± 0 , ± 10 , ± 20 , ± 30 and ± 40) in the zero tilt projection, γ increases after an optimal number of iterations is reached. (b) The effect of noise on the quality of the reconstruction is also evident in the coefficient of determination (COD), which continues to increase with increasing numbers of iterations in the absence of noise but decreases after an optimum is reached if noise is included. (c) For a given number of iterations, a higher COD and therefore a better solution is obtained for a larger angular ranges. (d) The COD is also slightly higher if a smaller angular increment is used to acquire the tomographic tilt series.

Figure 6.

Ratio of standard deviation in number of phosphorus atoms per pixel obtained from a SIRT reconstruction of a simulated volume containing no net phosphorus to the standard deviation obtained directly from the simulated volume ($\sigma_{\text{reconstructed}}/\sigma_{\text{simulated}}$) as a function of the iteration number. The simulated volume contained 30 slices in the x, y plane and the standard deviations were calculated for projections of the phosphorus distributions in the z -direction. Although the ratio $\sigma_{\text{reconstructed}}/\sigma_{\text{simulated}}$ increases towards unity for large numbers of iterations, for a typical reconstruction involving 30 iterations, the measured standard deviation from the SIRT reconstruction is a factor of 3 smaller than the standard deviation that was input into the simulation. The resulting curve can be used to correct the experimentally determined standard deviation obtained from SIRT reconstructions of elemental distributions in specimens of the same 80 nm thickness as used in the simulation.

Figure 7.

Measurement of thickness in three regions of sectioned Epon demonstrates shrinkage due to electron irradiation. Images in the left column are projections of a reconstructed STEM tomogram acquired with a total dose of $2 \times 10^3 \text{ e}^-/\text{nm}^2$, while the images in the right column are projections of the reconstructed tomogram after the specimen was exposed to a dose of $5 \times 10^7 \text{ e}^-/\text{nm}^2$, that we use to acquire 3-D phosphorus distributions. After exposure to the high electron dose, the sample shrinks to 43% of its initial volume. The elongation of the spherical gold particles in the z -direction is an artifact that is more evident in the single-axis WBP reconstruction, used here, than in the SIRT reconstruction.

Figure 8.

Images acquired from an 80 nm section of a *Drosophila* larval cell using automated EFTEM tomography; only data recorded at 0° tilt angle is shown. (a) Unfiltered TEM image, I^{unfilt} ; (b) zero-loss image, I^{zero} ; (c) 120 eV pre-edge image, I^{pre} ; and (d) 152 eV post-edge image, I^{post} above the P $L_{2,3}$ edge at 132 eV. (e) 2-D phosphorus distribution calculated with Eq. 4. Two regions of high phosphorus content are easily recognizable: cytoplasm containing ribosomes (*cyt*) and nucleus (*nuc*) containing areas of densely packed chromatin. The boundary separating the nucleus from the cytoplasm is shown as a dashed line.

Figure 9.

Volume rendered phosphorus distribution of two cells from a *Drosophila* larva. (a) Top view of the same cell shown in Fig. 8; (b) side view of selected region containing ribosomes (blue/green); (c) side view of region of nucleus containing densely packed chromatin (orange/red); (d) top view of another cell with region of cytoplasm containing ribosomes at upper right (blue/green) and nuclear chromatin (orange/red); (e) and (f) side views of region containing ribosomes and nuclear chromatin respectively; (g) section through the 3-D reconstructed volume demonstrating that the dual-axis SIRT algorithm preserves the spherical shape of the gold particles. The box enclosing a ribosome has dimensions of $7 \times 7 \times 7$ voxels, where the size of each voxel is 2.7 nm. White arrows indicate representative ribosomes in (b) and (e).

Figure 10.

EELS recorded at a beam energy of 300 kV from a 30 nm thick single crystal gold film in the region of the P L_{2,3} edge. It is evident that the broad delayed maximum in the spectrum from gold contributes strongly to the signal in the vicinity of the phosphorus edge, explaining why the gold fiducial particles are easily visible in the 3-D phosphorus images, enabling alignment of the EFTEM tilt series.

Figure 11.

Quantitative analysis of 3-D phosphorus distributions. (a) Histograms of phosphorus content of ribosomes and regions of Epon plastic embedding medium containing $7 \times 7 \times 7$ voxels; inset shows histogram of ribosomal volume (in voxels). (b) Histograms of phosphorus content in $7 \times 7 \times 7$ voxel regions of Epon and in similar sized regions of chromatin, with and without inclusion of the shrinkage factor. (c) Histogram of the number of phosphorus atoms in single voxels of Epon and chromatin.

Table 1

Average number of phosphorus atoms and statistical error determined experimentally from EFTEM tomography of a sectioned, freeze-substituted and Epon-embedded *Drosophila* larval cell in regions of embedding plastic, chromatin and ribosomes. Each voxel is 2.7 nm in size and 415 ribosomes were averaged.

	Plastic (P atoms) (7 × 7 × 7 voxels)	Chromatin (P atoms) (7 × 7 × 7 voxels)	Ribosomes (P atoms) (152 ± 28 voxels)
$\bar{n}$	-111	5789	7910
δn_{stat}	297	604	1871

Table 2

Summary of the statistical errors in 2-D and 3-D experimental data and theoretical predictions for an EFTEM phosphorus tomogram recorded at a beam voltage of 300 kV with a total dose of $5 \times 10^7 \text{ e}^-/\text{nm}^2$, a voxel size of 2.7 nm and an initial specimen thickness of ~120-150 nm.

	theory	experiment
$\delta n_{stat-2D}$	34 P atoms/pixel	116 P atoms/ pixel
$\delta n_{stat-3D}$	6 P atoms/voxel	20 P atoms/voxel