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Improved Z-axis Resolution in Serial Block Face SEM with Dual Primary Energies and Monte Carlo Simulation of Electron Scattering

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Serial block face scanning electron microscopy (SBF-SEM) provides nanoscale 3D ultrastructure of entire cells and tissue volumes. In SBF-SEM, an ultramicrotome built into the SEM specimen stage successively removes thin sections from a plastic-embedded, heavy metal-stained specimen. After each cut, the freshly exposed block face is imaged at a low incident electron energy using a backscattered electron detector to provide 3D ultrastructure with a resolution of approximately 5 nm in the plane of the block face and around 25 nm in the perpendicular z-direction, as limited by the slice thickness. We have explored the feasibility of improving the z-resolution in SBF-SEM by recording images at more than one primary beam energies, thus sampling different depths below the block surface [1,2].

A linear relationship was found between the depths of test structures, generated by Monte Carlo simulations [3], and the ratio of backscattered image intensities recorded at primary beam energies between 1.0 keV and 1.4 keV. An example model used for simulation is shown in Fig. 1a with two cuboids of size 50 nm x 50 nm x 12.5 nm containing a lead concentration of 3% (cuboid 1 and cuboid 2) and one of size 50 nm x 50 nm x 25 nm containing a lead concentration of 1.5% (cuboid 3). These three cuboids contain the same total amount of stain, but cuboid 3 has half the stain density of cuboids 1 and 2. As shown in Fig. 1b, we found that the electron probe penetrates ~13 nm and ~25 nm into the heavy-atom stained sample block at primary beam energies of 1.0 keV and 1.4 keV, respectively. This has enabled us to reconstruct the 3D model within a 25-nm surface layer at a z-resolution of around 12.5 nm, as shown in Fig. 3c. This result demonstrates that the matrix method is sufficiently sensitive to determine the depths of small stained features contained in a specimen block. Fig. 1d defines the noise level of the sub-slice reconstruction method. It is found that the lighter the stain, the noisier the reconstruction. We have combined this method with the serial block face cutting process. A Zeiss Sigma-VP SEM equipped with a Gatan 3View SBF system was used to acquire data.

An application of this method to a biology tissue is shown in Fig 2. An x-y view of the block face (Fig. 2a) shows a region of endoplasmic reticulum in a hepatocyte. An orthogonal view (Fig. 2b) shows a y-z plane in the same region imaged without sub-slice analysis and with the averaged intensities of the image stacks acquired at E_{low} = 1.0 keV and E_{high} = 1.4 keV, i.e., with the same dose used for sub-slice analysis. Despite some additional noise, it is evident that regions of ER, indicated by white arrows in the sub-slice image stack (Fig. 2c), appear sharp with one-pixel wide membranes that are well separated compared to the same structures in Fig. 2b. The signal-to noise ratio in the y-z plane can be improved by averaging several y-z images at different x-values. Five-slice averages along the x-axis of the y-z views in Figs. 2b and 2c are shown in Figs. 2d and 2e, respectively; the improvement in z-resolution obtained by sub-slice analysis for the ER membranes oriented close to the x-y plane is now seen more clearly [4].

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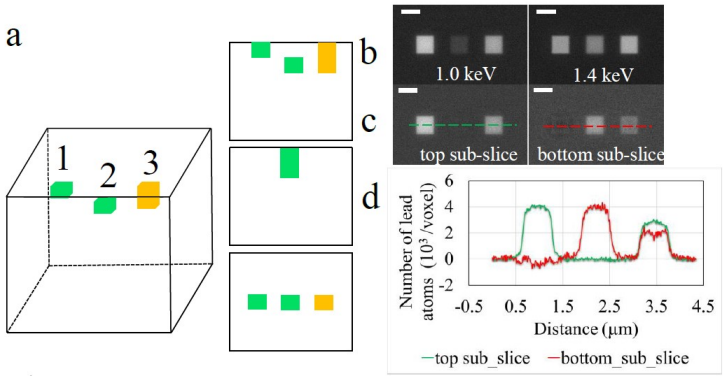

Figure 1. Monte Carlo simulation and calculated sub-slice specimen structure. (a) Three-dimensional geometrical model for two 50 nm x 50 nm x 12.5 nm stained cuboids (green) containing 3% lead, and one 50 nm x 50 nm x 25 nm stained cuboid (yellow) containing 1.5% lead, located at different depths in a 800 nm x 800 nm x 800 nm epoxy block, with views in x-z plane, y-z plane and x-y plane. Centers of cuboids are located 6.25 nm, 18.75 nm and 12.5 nm, respectively, from the top surface of the block (left to right). Dimension in z is not drawn to scale. (b) Simulated images at primary beam energies of 1.0 keV and 1.4 keV, respectively. (c) Calculated sub-slice specimen structure with a nominal z-resolution is 12.5 nm; scale bar, 50 nm. (d) Line profile of features in top and bottom sub-slice, as indicated by the dotted lines in (c).

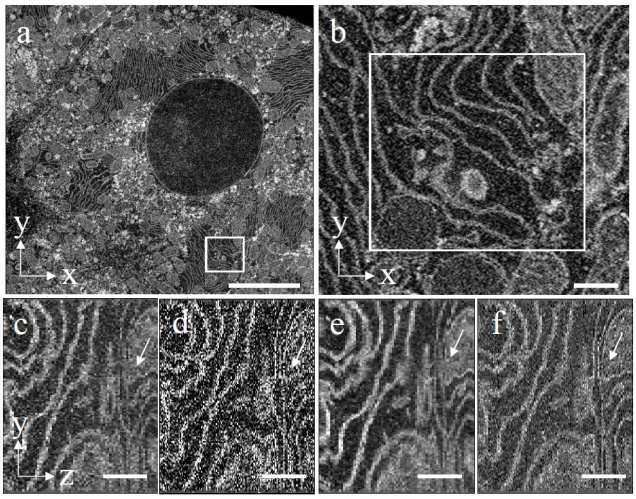

Figure 2. Reconstructed volume in x-y and y-z planes from liver sample cut at increments of 25 nm and imaged with primary energies of 1.0 keV and 1.4 keV. (a) Low-magnification BSE image of hepatocyte in the x-y plane acquired at primary energy of 1.4 keV. (b) Region indicated by square in (a) at higher magnification showing membranes of endoplasmic reticulum. (c) Average of 1.0 keV and 1.4 keV BSE image intensities displayed in the y-z plane. (d) Calculated sub-slice stack in the y-z plane showing improved spatial resolution relative to (c), but with additional noise. (e) Five-slice average in the x-direction of y-z view in (c). (f) Five-slice average in the x-direction of y-z view in (d). Arrows in (c–f) denote areas with improved resolution. Scale bar in (a), 5 μm; and in (b–f), 500 nm.

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