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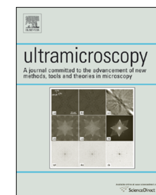

Whole-cell imaging of the budding yeast *Saccharomyces cerevisiae* by high-voltage scanning transmission electron tomography

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ABSTRACT

Electron tomography using a high-voltage electron microscope (HVEM) provides three-dimensional information about cellular components in sections thicker than 1 μm, although in bright-field mode image degradation caused by multiple inelastic scattering of transmitted electrons limit the attainable resolution. Scanning transmission electron microscopy (STEM) is believed to give enhanced contrast and resolution compared to conventional transmission electron microscopy (CTEM). Samples up to 1 μm in thickness have been analyzed with an intermediate-voltage electron microscope because inelastic scattering is not a critical limitation, and probe broadening can be minimized. Here, we employed STEM at 1 MeV high-voltage to extend the useful specimen thickness for electron tomography, which we demonstrate by a seamless tomographic reconstruction of a whole, budding *Saccharomyces cerevisiae* yeast cell, which is ~3 μm in thickness. High-voltage STEM tomography, especially in the bright-field mode, demonstrated sufficiently enhanced contrast and intensity, compared to CTEM tomography, to permit segmentation of major organelles in the whole cell. STEM imaging also reduced specimen shrinkage during tilt-series acquisition. The fidelity of structural preservation was limited by cytoplasmic extraction, and the spatial resolution was limited by the relatively large convergence angle of the scanning probe. However, the new technique has potential to solve longstanding problems of image blurring in biological specimens beyond 1 μm in thickness, and may facilitate new research in cellular structural biology.

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1. Introduction

Electron tomography (ET) is a method for obtaining nanometer-scale 3D reconstructions from tilt-series of images obtained by transmission electron microscopy (TEM) or scanning transmission electron microscopy (STEM). In biological applications, cellular ultrastructure and supramolecular assemblies can be visualized [1,2]. However, the penetrating power of electrons at typical accelerating voltages impedes the use of thick biological specimens. High-voltage electron microscopy (HVEM) was developed to extend the range of useful specimen thicknesses. Electrons accelerated by 1 MeV can penetrate to a depth of over 1 μm in biological samples embedded in resin [3]. Nevertheless, large numbers of multiply and inelastically scattered electrons are generated in thick specimens, thus degrading image quality [4].

Zero-loss energy filtering can remove inelastically scattered electrons [5], however, biological specimens in the micrometer thickness range transmit insufficient zero-loss electrons for a useful image. Most-probable-loss (MPL) imaging [6] can be used for improved imaging of thicker biological specimens. In this technique, images are formed by moving the energy-selection slit to the region of the energy-loss spectrum where majority of the (inelastically scattered) electrons can be found. MPL imaging has been successfully used to image Golgi-stained neuronal cells embedded in a resin section over 1 μm in thickness, using 300 keV electron microscope [6].

STEM has been shown to be an effective alternative technique for imaging thick biological specimens. In STEM, a focused electron probe is rastered over the specimen, and transmitted electrons are collected pixel-by-pixel on axial (bright-field) or off-axial (dark-field) detectors. These electrons have undergone multiple scattering and have lost energy, but since they need not be further imaged by electron lenses, image degradation due to lens aberrations does not

Abbreviations: STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy; HVEM, high voltage electron microscopy; BF, bright-filed; ADF, annular dark-field; ET, electron tomography; MPL, most-probable-loss; HAADF, high-angle annular dark-field

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occur [7]. STEM tomography was already used in biological applications over two decades ago [8], and is still being developed for use there [9–11]. It was also developed for inorganic materials, where high-angle annular dark-field (HAADF) STEM tomography was shown to be important [12]. HAADF STEM has been reported to provide improved contrast and better resolution with 1-μm-thick plastic-embedded biological specimens, compared to CTEM at the same accelerating voltage [10,11]. A small convergence angle (0.6 mrad) for the scanning probe, generated by a field-emission electron source, has been reported to provide excellent images, even from 1-μm-thick biological specimens embedded in plastic sections [13]. Until now, the increased penetrating power of 1 MeV electrons has not been combined with these benefits of STEM-mode imaging for thick biological specimens.

Budding yeast (*S. cerevisiae*) is a single-cell organism, typically egg-shaped and ~5 μm long and ~3 μm wide. We used this microorganism as a model in this work because of its small size and relatively simple organization. The intracellular structures of yeast have been investigated by semi-thin serial-section ET after high-pressure freezing and freeze-substitution [14,15], but serial-section ET is a laborious technique. Whole-cell subcellular morphology of yeast was also studied using soft X-ray tomographic imaging [16]; while easily able to accommodate a large-volume cell, the resolution of this technique is limited by the X-ray optics. The technique of serial-block-face scanning EM (SBF-SEM) has been shown to be very effective for making 3-D reconstructions of tissue and large cells [17–19]. A stained, resin-embedded sample is sectioned by a microtome inside the SEM chamber, revealing successive new block faces that are imaged by backscattered electrons to create a *z*-stack. The *x*–*y* resolution is as high as 5 nm, but the *z*-resolution is limited by the microtomed section thickness to about 25 nm. An alternative use of focused-ion-beam (FIB) milling before SEM imaging of each new block face [20,21]. While the block face has to be smaller than in SBF–SEM, this technique, known as "FIB tomography" yields nearly isotropic resolution, with *z*-resolution as good as ~10 nm. However, both of these techniques are completely destructive of the sample.

The benefits of HVEM tilt-series tomography are that the technique is non-destructive and the whole volume is seamlessly represented. The geometrical resolution in tilt-series electron tomography is discussed in Section 3.5, the effects of chromatic blurring are discussed in Section 3.1, and issues involved in thick-section STEM imaging are discussed in Sections 3.2 and 3.5.

In this study, we compared 1-MeV STEM tomography with 1-MeV TEM tomography for reconstruction of whole yeast cells, 3–5 μm in size, which were treated to clear the cytoplasm for better visibility of the major organelles. Tomograms resulting from STEM images showed improved contrast and finer definition of the major organelles, compared to tomograms from 1-MeV TEM images, in particular providing a clear "snapshot" of mitochondrial dynamics. Image resolution could be further improved if an FEG source was available, which would allow use of a probe convergence angle smaller than 20 mrad, which we used here. Nevertheless, we confirmed that HVEM STEM tomography gives the best possible results for ET of thick biological specimens, and has good potential for applications in cellular structural biology.

2. Materials and methods

2.1. Yeast cells and specimen preparation

In this study, we used a haploid strain of the budding yeast *S. cerevisiae* (the yMEE13-based Δ*vms1* strain) [22,23]. Samples were prepared by post-treatment with potassium permanganate ($KMnO_4$) to extract soluble components [24]. Cells were cultured in a galactose medium at 30 °C for 12 h until the optical density at 600 nm reached ~1, and then harvested by gentle centrifugation. The cells were chemically fixed with 2% glutaraldehyde (pentanedial) in pH 6.8 phosphate buffer for 2 h, cooled on ice. The fixative was removed by washing with the buffer solution, and the sample was treated with 1.5% aqueous $KMnO_4$ for 4 h at 4 °C, then rinsed several times with distilled water. The samples were then dehydrated through a conventional alcohol series and infiltrated with an epoxy resin (Epok 812; Oken Shoji Co., Tokyo, Japan). The resin was cured at 60 °C for two days and the resulting sample blocks were sectioned with an Ultracut S ultramicrotome (Reichert, Vienna, Austria) to a thickness of 3–4 μm. The sections were mounted on an oyster-type square grid (Stork Veco BV, Eerbeek, Netherlands). No post-staining of the sections was carried out. 25-nm colloidal gold beads were deposited on both sides of the sections to serve as fiducial markers.

2.2. Electron tomography

Tilt-series images were recorded at an accelerating voltage of 1 MeV on a JEM-1000K RS HVEM (JEOL Ltd., Tokyo, Japan) [25]. Conventional TEM bright-field (BF) images were recorded on a mirror-coupled 2k × 2k CCD camera (Orius SC200; Gatan Inc., Warrendale, PA, USA). STEM images were collected by using two electron detectors. For axial bright-field (BF) imaging, a detector 8 mm in diameter was located 300 mm below the projector lens. The BF electrons passed through 4 mm central hole in an annular dark-field (ADF) detector, which was located 184 mm below the projector lens, and collected the electrons that were scattered at higher angles. Images from both STEM detectors were simultaneously recorded. Tilt series of yeast cells were collected at 2° angular interval from −60° to +64° (the full range of ±70° was limited by specimen occlusion). The imaging doses on the specimen for each tilt image were 1.3 $e^-/Å^2$ in TEM and 1.2 $e^-/Å^2$ in STEM, respectively. However, the tilt series was collected manually, with tracking and focusing on-axis. Thus, the total electron dose per tilt image was approximately ~2.0 $e^-/Å^2$ in TEM and ~1.5 $e^-/Å^2$ in STEM, and the total electron dose for the tilt series was approximately ~126 $e^-/Å^2$ in TEM and ~94.5 $e^-/Å^2$ in STEM. A complete tilt series in STEM mode was done, followed by a second tilt series of the same cell in TEM mode.

Image alignment and 3D reconstruction were carried out by using IMOD [26], and image segmentation was performed with Amira (FEI Visualization Science Group, Burlington, MA, USA). To estimate the thicknesses of the specimen before and after collecting each tilt series, we recorded TEM images of the section tilted at ±45° and compared the projected distances between gold particles on the top of the section and one on the bottom. A trigonometric function was then used to calculate the section thickness.

3. Results and discussion

3.1. Chromatic blurring in thick specimens

Fig. 1 shows the energy-loss spectrum from ~3-μm-thick resin section containing whole budding yeast cells, imaged at 1 MeV. The zero-loss peak is insignificant, and there is a broad energy spectrum centered around 200 eV, due to inelastically scattered electrons. Because the energy distribution is very broad, chromatic aberration in the objective lens caused blurring of the image. The amount of blurring due to chromatic aberration is formulated as

$$\delta = C_c \frac{\Delta E}{E} \beta \quad (1)$$

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