Reports

High-resolution three-dimensional reconstruction of a whole yeast cell using focused-ion beam scanning electron microscopy

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We developed an approach for focused gallium-ion beam scanning electron microscopy with energy filtered detection of backscattered electrons to create near isometric voxels for high-resolution whole cell visualization. Specifically, this method allowed us to create three-dimensional volumes of high-pressure frozen, freeze-substituted *Saccharomyces cerevisiae* yeast cells with pixel resolutions down to 3 nm/pixel in x, y, and z, supported by both empirical data and Monte Carlo simulations. As a result, we were able to segment and quantify data sets of numerous targeted subcellular structures/organelles at high-resolution, including the volume, volume percentage, and surface area of the endoplasmic reticulum, cell wall, vacuoles, and mitochondria from an entire cell. Sites of mitochondrial and endoplasmic reticulum interconnectivity were readily identified in rendered data sets. The ability to visualize, segment, and quantify entire eukaryotic cells at high-resolution (potentially sub-5 nanometers isotropic voxels) will provide new perspectives and insights of the inner workings of cells.

Elucidating the three-dimensional (3D) spatial distribution of structures and molecules within cells and tissues is critical for determining the nature of numerous cellular processes. Consequently, a multitude of technologies using photons and electrons (1–3) as well as x-rays (4) have been developed to permit the 3D spatial visualization of organisms, tissues, cells and sub-cellular constituents. In particular, focused ion beam scanning electron microscopy (FIB-SEM) has drawn significant attention as a powerful technology for creating 3D volumes of biological specimens at electron microscopy resolution (5,6). To date, the majority of FIB-SEM approaches have employed variations in conventional chemical fixation that include an aldehyde primary fixation in combination with osmium tetroxide and uranyl acetate *en bloc* staining (6,7) while others (8,9) also included thiocar-

bohydrazide-osmium ligand treatment (10), potassium ferrocyanide (9,11–14), and/or tannic acid (5, 14) for enhanced contrast. In one instance, pre-fixation in paraformaldehyde prior to cryo-preservation with high-pressure freezing was employed, but no differences were observed compared with conventional fixation, thus cryo-data was not shown (15). FIB-SEM of biological samples has been demonstrated at ~50–60 nm (5,9,13–14), 30–40 nm (11) and down to ~18–20 nm slice z-intervals in mammalian cells (6,15,16) and 100 nm z-interval specifically in yeast (7) which are comparable to serial sectioning methods or block-face imaging using diamond knives (1,17). More recently, the limits of FIB-SEM has been even further pushed with FIB conditions set to acquire 5 nm isotropic voxels in brain tissue (12). For high-resolution 3D imaging of biological samples, transmission electron microscopy

(TEM) tomography is the method of choice for acquiring cellular structures down to ~3 nm resolution (18), including yeast (19); FIB milling in conjunction with TEM tomography has been shown as a powerful enhancement to cryo-TEM tomography (8). However, TEM tomography is typically limited to 0.5 micron thick sections, making reconstruction of an entire eukaryotic cell technically very challenging. In an attempt to approach the resolution of TEM tomography using FIB-SEM for whole cell reconstructions, we pushed the limits of focused ion beam milling and backscattered electron (BSE) detection to obtain ca. 3 nm isotropic voxels. This significant z-resolution improvement enabled us to create high-resolution 5 nm x 5 nm x 5 nm isotropic renderings and quantification of an entire cell, including numerous subcellular structures, with the yeast model organism, *Saccharomyces*

cerevisiae. The purpose of this work was to optimize cryo-fixation sample preparation, milling and imaging conditions to quantify and document the 3D spatial distribution of sub-cellular structures from an entire single cell at nanoscale resolutions.

Materials and methods

Cell growth

S. cerevisiae yeast strains BJ5464 and BY4742 grown in YPD were used in this study. Overnight cultures were grown to mid-log phase ($0.8 \leq OD_{600} \leq 2.0$) in 500 mLs – 1 L of YPD media (30°C , 275 rpm) (20–21). YPD medium was prepared as described (22). To contrast the effects of cellular stress induced by chemical treatment (23), 8 mM of dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA) was added to BY4742 (5 nm isotropic data set only). Following incubation for 2 h at 30°C and 275 rpm, unstressed and stressed BY4742 cells were harvested. Cells were centrifuged at 2,500 rcf for 5 min at 4°C for subsequent cryo-preservation.

Electron microscopy sample preparation

A yeast paste was high pressure frozen in a Leica EMPact in 1.2 mm x 200 μm hats and freeze substituted at -80°C in 2% osmium tetroxide in acetone containing 1% water for 3–4 days as described previously (24). It must be noted, that even with freeze-substitution, some yeast membranes are difficult to contrast (25). Samples were warmed to -20°C over a 16 h period, to 4°C for 3 h, to room temperature for 2h and washed in acetone. Yeast were *en bloc* stained in saturated uranyl acetate in 100% acetone overnight, washed in acetone, transferred to 1:1 ethanol:acetone and *en bloc* stained with saturated lead acetate in 1:1 ethanol: acetone for 2–4 h (26). Samples were dehydrated in acetone and infiltrated with Embed-812 epoxy resin, embedded in BEEM capsules and polymerized for 48 h at 60°C . All samples were evaluated on a Zeiss LIBRA 120 Plus transmission electron microscope with a Gatan Ultrascan 1000 2k x 2k camera prior to FIB analysis.

FIB/SEM image stack acquisition

Cured resin blocks were manually trimmed to expose yeast cells and a glass knife was used to face the block. The final block size was trimmed as small as possible (about 1 mm in size) so that sample drift from continuous degassing of resin was minimized. The block was then attached to an aluminum SEM stub with silver paint. The block and stub was coated with Pt for 5 min resulting in a continuous conductive Pt

layer about 100 nm thick. This continuous Pt layer served as a medium for dissipating charge and heat generated from the electron and gallium ion beams interacting with the sample block. A Zeiss Auriga FIB/SEM XBeam system that is equipped with a Cobra-focused gallium ion beam column, a Schottky field emission gun and a Gemini electron column was employed for all the milling and imaging work in this study. For imaging and milling, the sample was tilted to a 54° angle and placed at a working distance about 5 mm below the SEM final lens so that the sample surface was perpendicular to the ion beam where the electron beam and ion beam meet. The electron beam (SEM imaging) was used to locate a site of interest and then the ion beam (FIB) was used to prepare the block face for automated image stack acquisition. For FIB milling, we employed a 30 keV Ga ion beam with a 12 nA current to cut a trench so that the 3D imaging targeted volume was exposed and the surface was prepared to be milled and imaged. This also helped accommodate some re-deposited materials and reduced the possibility that neighboring materials would interfere with the passing of BSEs to the detector. To reduce the damage caused by large current FIB milling, the periphery of the volume of interest was cleaned with a 1 nA beam. A 600 pA beam was used for final 3D data acquisition.

Image stack acquisition was executed in an automated fashion where both ion milling parameters (i.e., probe current, dwell time and milling interval (z-interval)) and SEM imaging parameters (i.e., beam energy and current, dwell time, pixel size, detector selection and energy filtering grid bias, focus and stigmator settings) were set before the process started. Specifically, all images were taken with a beam energy at 1.5 keV, and probe current of 931 pA (60 μm aperture and high current mode), working distance at 5.0 mm and tilt angle at 54° . Line averaging was used for noise reduction ($N = 30$) at an image store resolution of 3073×2304 with a dwell time of 12.7 $\mu\text{sec}/\text{pixel}$, resulting in a total cycle time of around 1.5 min/image. Electron dosage per square nanometer was calculated at total electrons per square nanometer (30 line passes in “line average”), or electrons per square nanometer per pass. For the 3 nm data set, it was 8220 electrons/ nm^2 and 274 electrons/ nm^2/pass , the 5 nm data set 2958 electrons/ nm^2 and 99 electrons/ nm^2/pass and the 15 nm data set 329 electrons/ nm^2 and 11 electrons/ nm^2/pass . The energy filtering grid was set at 500 eV, 750 eV and 1000 eV for the data sets with 15 nm, 5 nm, and 3 nm z-interval, respectively. A typical stack of images with an x-y-z pixel size of 5

nm covered a volume of $\sim 8 \mu\text{m} \times 10 \mu\text{m} \times 8 \mu\text{m}$ and took about 35 h.

Normal FIB artifacts such as curtaining were minimized by careful preparation of the block face and deposition of surface protection layer (Pt or C). Surface charging was also reduced by recoating the sample after a trench was FIB-cut (making the side walls of the cut block conductive) and by using the energy filter to block the lower energy BSEs (more susceptible to surface charges) from reaching the Energy Selective Backscattered electron (EsB) detector. We also noted that blanking the electron beam during FIB milling allowed us to obtain higher quality images. Through long acquisitions hours, image drift was an unavoidable phenomenon and was minimized by the following steps: (i) we cut the embedded sample block as small as possible (~ 1 mm cube) and pre-evacuated the sample for at least 24 h before starting FIB sectioning and imaging, (ii) we mounted the sample on a stub with silver paint around it, (iii) we coated the sample with about 10–15 nm thick Pt layer using a Cressington Sputter Coater to make the surface as conductive as possible in order to avoid surface charge induced image drift, and (iv) we allowed at least one hour for the sample to achieve equilibrium (mechanically and thermally) after the sample was inserted and the site of interest was found.

Stack alignment, segmentation, and 3D presentation

Once an image stack was acquired, alignment of the image stack (See Supplementary Material for additional details), noise reduction, reconstruction of a voxel image from the aligned image stack, segmentation based on intensity and/or shape of known organelle structure and the final presentation in 3D space was generated. For the cell FIB sectioned at a 10 nm isotropic pixel resolution, the ER was segmented with Avizo software application framework whereas all other organelles were segmented in IMOD (5,27). All other data sets were processed exclusively using Avizo with a customized workflow supporting individual cell segmentation. Segmentations were based on intensity and known morphology of the target structure. Segmentation results were used to reconstruct the geometrical representations of the organelles and to extract quantitative information. Avizo was also used to generate all pictures and movies.

Monte Carlo simulations

When a primary electron beam penetrates into solid sample surface, backscattering occurs. The BSEs carry energy below the

Figure 1. Enhanced FIB resolution. (A) Transmission electron micrograph of *Saccharomyces cerevisiae*. Scale bar equals 500 nm. (B) Comparable SEM micrograph. Scale bar equals 500 nm. (C) XZ cross-section of a ~3 nm isotropic FIB data set. Scale bar equals 500 nm. (D) Magnified view of boxed area (black) from panel (C). Arrows indicated nuclear pores. Scale = 500 nm. (E) Left panel shows an XZ view of a 61 nm vesicle at 3 nm z-interval. Right panels show 18 serial XY images at 3 nm z-interval through the vesicle. Scales = 100 nm. (F) Left panel shows XZ view of a 74 nm vesicle at 15 nm z-interval. Right panels show 6 serial XY images at 15 nm z-interval through the vesicle. (G) Monte Carlo simulation of electron trajectory within epoxy with 1% (atom) Os at median energy (250 eV) of the energy filtering window: 500 eV, primary beam energy (E_p): 1500 eV, filtering grid bias (E_{grid}): 1000 eV, sample tilt (T): 54°, 500 electrons. (H) Electron trajectory without tilt or energy filter - parameters used same as in (G), but calculated at median energy at 750 eV and T = 0°.

primary beam energy due to inelastic interactions between the incident electrons and the solid sample. The distance that the BSEs travel within the sample back toward the surface follows nearly a linear relationship with the energy they carry (28,29) and can be calculated with Monte Carlo simulation using CASINO v2.4.8.1. In this work, the primary beam energy was set at 1500 eV and a bias energy was applied to the energy filtering grid thus preventing BSEs

of energy below the set bias (for example, 1000 eV) to reach the detector (Supplementary Figure 2). Therefore, only BSEs carrying energy above the set bias energy reached the detector. We conservatively calculated the stopping range of electrons in the simulated yeast sample [Epoxy resin ($C_{21}H_{25}ClO_5$) with 1% (atom) Os] at energy filtering biases of 1000 eV and 0 eV (no filter, conventional BSE detection system) respectively, using median values of

the selected energy windows, for instance, 250 eV for the set bias of 1000 eV (e.g., energy window width of 500 eV) and 750 eV for no filter (e.g., primary beam energy, 1500 eV). Figure 1g showed the electron trajectory at 54° tilt with 1000 eV bias and Figure 1h showed the electron trajectory when no filtering was applied at 0° tilt.

Results and discussion

To achieve optimal morphology and contrast by EM, *S. cerevisiae* cells were high-pressure frozen, freeze-substituted and *en bloc* stained in lead and uranyl acetate prior to epoxy resin infiltration. To our knowledge this is the first demonstrated report of FIB-SEM resin infiltrated 3D cellular imaging using cryo-preservation without conventional pre-fixation/fixation. While *en bloc* lead staining (26) has been employed for serial block face imaging (30) our procedure demonstrated, as expected, excellent contrast via FIB-SEM. A comparison between TEM (Figure 1a) and BSE images from FIB-SEM (Figure 1b) showed the images to be very similar, but with somewhat reduced image detail and increased contrast of yeast cell walls with FIB-SEM. We then acquired serial FIB slices of yeast cells at 15 nm, 5 nm, and 3 nm z-intervals, which were carefully chosen to assess the practicalities, limitations and impact these parameters had on image quality and structural integrity. While the 15 nm z-interval produced sufficient detail for many studies and the 3 nm z-interval provided the maximum spatial resolution of cellular components, 5 nm z-interval data was a reasonable balance of resolution, data size and the time required to collect data for an entire cell. The data set acquired at the 3 nm z-interval resulted in an XZ image (Figure 1c) that compared favorably to the XY FIB-SEM image (Figure 1b). A close inspection of the XZ image (Figure 1d) showed that relatively small structures, such as nuclear pores (arrows), were easily identified.

We report here, the ability to image through large cell volumes at reduced z-intervals down to 3 nm isotropic voxels, which is a 6-fold reduction in z-interval from several previous reports using FIB-SEM in cells (6,7,15) and comparable to a recent 5 nm isotropic FIB-SEM data set acquired from brain tissue (12). Our data extended upon this previous work by reducing the isotropic resolution to 3 nm and segmenting and quantifying numerous major cellular organelles from a single cell at 5 nm isotropic resolution using cryo-preservation. Since it was not possible for us to directly measure the 3 nm milling as defined via software acquisition settings, we

used three primary methods to validate the approach. First, as a rough visual inspection, the data showed no conspicuous distortions in aspect ratio when viewing the XY versus Z axis (Supplementary Video 5). Second, in order to empirically confirm the z-interval of ~ 3 nm, we used spherical vesicles within the resin embedded cell as an internal and sample specific control with a previously described cylindrical diameters method for measuring mean section thickness of TEM (31) and 3D FIB data (6,11). Our results demonstrated a mean slice thickness of 3.603 nm, (SD = 0.606, N = 30). Consistent with this result, using $3\text{ nm} \times 3\text{ nm} \times 3\text{ nm}$ isotropic voxels, a 61 nm vesicle spanned 18 slices (Figure 1e). In comparison, the 15 nm z-interval data set required 6 slices to image section through a 74 nm diameter vesicle (Figure 1f). Third, we assessed milling consistency via continuous playback of the stacks of images (Supplementary Video 6) and while not flawless, overall the data showed relatively smooth transition from section-to-section for the data set. Furthermore, the gradual change in vesicle diameter at each step (Figure 1e) suggested that the BSEs that reached the EsB detector were restricted to a few nanometers from the surface. When low energy electrons hit a specimen, the penetrated distance depends roughly linearly on the electron's energy. The same is true with BSEs (8,29) exiting the specimen surface which have a wide distribution in angle and energy since they may have experienced multiple scattering events and originated from different depths in the specimen. Conventional BSE detectors mounted below the objective lens typically collect nearly all BSE exiting the specimen surface and as a consequence x-, y-, and z-resolution are reduced. To overcome these limitations, an in-column EsB detector was employed (Supplementary Figure 1) similar to a previous report (12). BSEs with large scattering angles or that originated deep within the sample were restricted from reaching the EsB detector either geometrically or by applying a bias energy at a filtering grid before the detector (Supplementary Figure 2). In this study, for 3 nm and 5 nm z-interval data, the primary beam energy was set to 1500 eV and the energy filtering grid bias was set to 1000 eV, thus only BSEs having lost less than 500 eV were allowed to pass through the grid and reach the EsB detector.

Modeling electron-sample interactions of biological systems is highly complex due to local chemical heterogeneity, including variations in structure and composition and the lack of *a priori* knowledge of the detailed cellular composition of our samples. Though the BSE radius, depth of

Figure 2. 3D segmentation. (A) 3D 3.72 nm $\times$ 3.72 nm $\times$ 15 nm anisotropic FIB data set showed endoplasmic reticulum (ER; yellow), nucleus (light blue), cisternae (green), vesicles (light green), lipid droplets (orange), microtubules (pink), mitochondria (red) and cell wall (gray). (B) 3D segmentation of ER (yellow), mitochondria (red) and nucleus (blue) of a whole yeast cell at 5 nm isotropic voxels. Cell diameter 3.5 μm . (C) 3D segmentation of 5 nm isotropic data set with ER (yellow) and mitochondria (red) interconnectivity (arrows). (D) 3D 3 nm $\times$ 3 nm $\times$ 3 nm isotropic FIB data set showed, nucleus (light blue), nuclear pores (dark blue), cisternae (green), vesicles (light green), and mitochondria (red). Cell diameter 3.2 μm and final data set volume of 25.56 μm^3 . (E) 3D segmentation of nucleus (light blue), nuclear pores (dark blue) and heterochromatin (bright blue) at 3 nm isotropic voxels. Nucleus diameter 1.2 μm . (F) 3D segmentation of plasma membrane (light pink) and plasma membrane invaginations (arrows).

emitted BSEs and BSE coefficients have been modeled previously in two pure metals (carbon and osmium) for 2 keV beam with tilt (9), it is far from the real conditions of resin infiltrated biological cell systems. Here, we employed a simple and conservative composition, epoxy resin and osmium fixative [$\text{C}_{21}\text{H}_{25}\text{ClO}_5$ + 1% (atom)Os], to estimate the extent

of electron penetrations within the epoxy embedded yeast. Monte Carlo simulation of electron-sample interactions showed that the BSEs for imaging were predominantly 3–4 nm below the surface ($E_0 = 1500\text{eV}$, $E_{\text{grid}} = 1000\text{eV}$, Tilt = 54°) (Figure 1g) as compared with over 20 nm in the case of conventional BSE detection for FIB without energy selection ($E_0 = 1500\text{eV}$,

Table 1. Quantitative Analysis of Cellular Components.

Cellular component	Volume (μm^3)	Volume Percentage	Surface Area (μm^2)
Endoplasmic reticulum	0.420643	2.2%	31.403
Nuclear envelope	0.227859	1.2%	11.416
Heterochromatin	0.577432	3.0%	24.441
Euchromatin	0.459362	2.4%	20.716
Golgi	0.022677	0.1%	1.291
Mitochondria	0.299339	1.6%	6.949
Lipid droplets	0.139214	0.7%	2.692
Vesicles	0.000256	0.0%	0.025
Vacuoles	1.480174	7.8%	27.164
Cell Wall	3.017192	15.9%	67.115
Quantitative analysis of volume, volume percentage and surface area of cellular components segmented in Avizo software.			

Tilt = 0°) (Figure 1h). This simulation confirmed the empirical results (Figure 1e) where the images of 3 nm-interval slices consisted of mainly BSEs from those respective slices. The addition of atomic lead and uranium as part of simulations would only serve to further reduce the BSE signal collection volume (i.e., less than 3 nm). Considering the complexity of biological samples and our inability to reliably determine the local heavy metal stain concentration, simulations with lead and uranium were not included, thus we emphasize our model estimates of electron penetration under the conditions employed were intended to be conservative.

As part of our characterization of the FIB-SEM high-resolution cell imaging approach, we sought to acquire, render and compare yeast volumetric data sets with 15 nm (Figure 2a), 5 nm (Figure 2b, c) and 3 nm (Figure 2d-f) z-intervals. FIB-SEM acquisition produced very large data sets and in the case of the 5 nm experiment, the resulting 3D volume was a $\sim 10 \times 7.5 \times 7.5 \mu\text{m}^3$ and contained fine structural details of several yeast cells represented by about 5 billion voxels. This large amount of information was examined interactively with state-of-art graphics processing unit (GPU) accelerated volume rendering technology (Supplementary Figures 3, 4 and 5). Whole cells were extracted from the volume by creating a mask that isolated region of interests (Supplementary Figure 4). Detailed biological structures within the cell were segmented using a combination of automatic and interactive software algorithms prior to reconstruction. The 15 nm z-interval data set of a budding yeast cell with selected segmented organelles revealed excellent structural and positional detail; however, the z-axis stepping artifact of a spherical nucleus (light blue) and

other structures was prominent due to the anisotropic resolution (Figure 2a, Supplementary Video 1). A 5 nm isotropic voxel data set of an entire yeast cell (Figure 2b, Supplementary Video 2) showed significant improvement in overall structural detail (i.e., mitochondria, endoplasmic reticulum, nucleus) with concomitant reduction in z-axis stepping artifacts (i.e., nucleus) in this camera perspective. Furthermore, the spatial relationships of organelles with each other as well as sites of endoplasmic reticulum and mitochondrial interconnectivity (32) were readily documented (Figure 2c, Supplementary Video 3). To further push the systems capabilities while dramatically extending the total cell volume visualized, we acquired a data set at 3 nm isotropic voxel settings (Figure 2d) which permitted high-resolution 3D reconstructions of the nucleus, nuclear pore position and nucleoplasm/heterochromatin (Figures 2d and 2e), cisternae and vesicle distribution (Supplementary Video 4) and plasma membrane sights of invagination termed eisosomes (33) (Figure 2f). Images used in Figure 1c-f were derived from this same 3 nm volumetric data set

and the raw x-y aligned but unsegmented stack showed overall structural detail in the x-y and x-z axis with our approach (Supplementary Video 5). The accurate reconstruction of organelles in an entire cell allowed us to quantify targeted cellular structures, including but not limited to volume, volume percentage and surface area of various organelles (Table 1).

We conclude that this quantitative high-resolution FIB-SEM approach will open many new possibilities for visualizing the cellular organization of different cell and tissue types and complement other existing high-resolution approaches such as TEM tomography (19) and serial block face imaging (17). The segmentation of selected organelles and cellular components revealed their 3D spatial relationship with unprecedented z-resolution by FIB-SEM, approaching the gold standard of TEM tomography. Furthermore, the determination of organellar inter-relationships and connectivity (32,34), improvements in nano-scale volumetric quantitative measurements/comparisons as well as the generation of targeted sub-cellular molecular distribution/localization maps using correlative microscopy (35) including photooxidation (36) are natural extensions of this methodology. Our overall goal to optimize conditions for high-resolution 3D reconstructions of entire single cells allowed us to quantify and understand the spatial distribution of numerous sub-cellular structures (Figure 2, Table 1). For the greatest accuracy of volumetric measurement with 3D FIB data sets, reduced milling intervals provide a distinct advantage (37) and for the yeast cell features, we found that this goal was best achieved by acquiring isotropic pixels at ~ 5 nm or less. As with any approach which involves significant sample preparation and image processing (i.e., fixation, dehydration, resin embedment, image acquisition and analysis routines) consideration must be given to artifacts and

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limitations of each and interpretation adjusted accordingly. While *S. cerevisiae* served as a challenging biological specimen for optimizing sample preparation and FIB-SEM validation, we see no technical limitation for extending this strategy to larger cells at comparable pixel resolutions. However, significant improvements in FIB-SEM 3D resolution may likely require a technological breakthrough in SEM resolution itself, as well as improvements in sample stabilization and other environmental influences in the microscope and/or a method for tracking and correcting sample drift.

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Competing interests

Dongguang Wei is an employee of Carl Zeiss Microscopy, LLC a manufacturer of the Auriga 60 FIB-SEM used in this study. Shuang Zhang is an employee of Visualization Sciences Group, Inc. a developer of Avizo, the high-performance visualization software framework used in this study. All other authors declare that there is no conflict of interest.

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