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Quantitative 3-D imaging of eukaryotic cells using soft x-ray tomography

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Abstract

Imaging has long been one of the principal techniques used in biological and biomedical research. Indeed, the field of cell biology grew out of the first electron microscopy images of organelles in a cell. Since this landmark event, much work has been carried out to image and classify the organelles in eukaryotic cells using electron microscopy. Fluorescently labeled organelles can now be tracked in live cells, and recently, powerful light microscope techniques have pushed the limit of optical resolution to image single molecules. In this paper we describe the use of soft x-ray tomography, a new tool for quantitative imaging of organelle structure and distribution in whole, fully-hydrated eukaryotic *Schizosaccharomyces pombe* cells. In addition to imaging intact cells, soft x-ray tomography has the advantage of not requiring the use of any staining or fixation protocols—cells are simply transferred from their growth environment to a sample holder and immediately cryofixed. In this way the cells can be imaged in a near native state. Soft x-ray tomography is also capable of imaging relatively large numbers of cells in a short period of time, and is therefore a technique that has the potential to produce information on organelle morphology from statistically significant numbers of cells.

Keywords

Cellular imaging; microscopy; organelle characterization; *Schizosaccharomyces pombe*; soft x-ray tomography; segmentation; yeast

1. Introduction

Imaging is fundamental in all biological and biomedical research, and the essence of cell biology. In the years since George Palade, Keith Porter and Albert Claude (Claude, 1949; Palade and Porter, 1954) first used electron microscopy to visualize the ordered compartments

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of eukaryotic cells, an enormous amount of effort has been devoted to imaging and classifying organelles (Subramaniam, 2005). Electron microscopy has remained the primary imaging tool for this type of study. However, the relatively shallow penetration depth of electrons means that eukaryotic cells can only be imaged after they have been fixed and sectioned to be less than 0.5 μm thick (McDonald, 2007). Sectioning is frequently a frustrating and time consuming process, and requires the use of protocols—such as plastic embedding—that have the potential to significantly impact the fidelity of the cell ultrastructure (Perktold et al., 2007). In this paper we present a method for quantitatively imaging the organelles in whole eukaryotic cells using soft x-ray microscopy. By using x-rays with wavelengths in the ‘water window’ the resultant 3-Dimensional reconstructions of the cell have excellent natural contrast (Gu et al., 2007; Larabell and Le Gros, 2004). Consequently the cells are not exposed to potentially damaging staining or fixing protocols prior to imaging with this technique.

Most molecular interactions that occur in a normally functioning eukaryotic cell are location specific. Consequently, a number of methods have been developed to track the precise location of proteins inside the cell. In this regard, genetically encodable Fluorescent Proteins (FP) have been phenomenally successful, and have been developed for a wide range of applications, including measuring the distance between differently labeled molecules (Giepmans et al., 2006) and as a means of obtaining relatively high spatial resolution localization information (Betzig et al., 2006; Gustafsson, 2005; Hell, 2007; Rust et al., 2006). That said, FP-based imaging methods have an obvious shortcoming—they can only image the fluorescent signal provided by the tagged molecules, so no information is obtained on the internal organization of the cell. Learning about the internal organization requires the use of a complimentary imaging technique, typically Electron Microscopy. It has become commonplace to determine the location of a particular protein in a cell using the fluorescence signal from a FP tag, and use this knowledge as a guide for collecting higher resolution images using electron microscopy (Sosinsky et al., 2007). While the approach of using complementary imaging techniques provides valuable insights into the details of cellular processes, it would be preferable to obtain this information using a single imaging method. Soft x-ray microscopy has the demonstrated potential to meet this need. For example, in previous work we have already demonstrated the practicality of using electron dense labels to localize proteins in mouse 3T3 cells (Meyer-Ilse et al., 2001). In this paper we show the ability of soft x-ray tomography to examine the structural composition of cells, which is critical in order to extend protein localization studies into three dimensions.

In addition to imaging cells that are minimally perturbed, a further strength of soft x-ray tomography is the throughput; a complete set of projection images can be collected in less than three minutes. The throughput is further enhanced in the case of yeast because a number of cells are in the field of view at the same time. This means that each data collection run results in tomograms of three to five cells. Consequently, cells can be imaged in statistically significant numbers. This allows for an accurate correlation of phenotypic and morphological changes in the cellular structure with genetic and biochemical data. This is of particular importance for studies focused on subtle changes in organelle morphology.

In many types of cells, the number and organization of certain organelles can change quickly in response to environmental factors such as cell density, temperature, oxygen tension, and the availability of nutrients (Conibear and Stevens, 1995; Egner et al., 2002; Weisman et al., 1987). Yeast is a particularly good model system for studying the size, shape, and distribution of organelles such as mitochondria as a means of coping with changes in their environment (Anesti and Scorrano, 2006; Hales, 2004; Hermann and Shaw, 1998; Hoog et al., 2007; Jakobs et al., 2003; Jensen, 2005; Logan, 2003; Logan, 2006; Sun et al., 2007; Yaffe et al., 1996). For example, during logarithmic growth the mitochondria in yeast constantly undergo fusion and fission. If the cells exhaust the available nutrients, the cells enter a stationary phase

characterized by cell cycle arrest and other specific physiological, biochemical, and morphological changes. One such change is the cessation of mitochondrial fission, resulting in unbalanced fusion and the formation of ‘giant’ mitochondria. Yeast in the stationary phase can remain viable in this state for very long periods of time. When their environment becomes more suited to growth, yeast in the stationary phase can quickly revitalize and resume the normal cell cycle, including metabolic processing. As a consequence, when cells move from the stationary phase to logarithmic growth, the size, location and mobility of the mitochondria rapidly reverts to that typically seen in cells growing logarithmically.

Yeast vacuoles also respond to environmental changes, in particular changes in osmolarity. When fission yeast cells, *Schizosaccharomyces pombe*, are taken from media and placed in water, for example, the smaller vacuoles undergo rapid fusion to form much larger vacuoles (Bone et al., 1998).

In the work described here we demonstrate the power of soft x-ray tomography by quantifiably determining the size, shape and organization of *Schizosaccharomyces pombe* organelles in stationary phase cells using soft x-ray tomography. In particular, we quantified and characterized the mitochondria and vacuoles in cells that had become stationary during different stages of the cell cycle—freshly budded daughter cells, single mature cells, and mother-daughter cells. This work follows our previous research on *S. pombe* (Gu et al., 2007), in which we studied the process of cell division.

2. Materials and Methods

Cell culture

Wild type *S. pombe* cells (strain #972 h-) were grown with rotary shaking at 30°C in YES media (yeast extract + adenine + casamino acids) supplemented with leucine, histidine, uracil and dextrose.

Light microscopy

Mitochondria and vacuoles were stained by adding fluorescent dyes to cells undergoing logarithmic growth. These stains had no apparent effect on cell growth when compared with unstained controls (data not shown). The dyes remained active for upwards of 72 hours after incubation with cells, and images taken after long incubation appeared very similar to images taken at the cessation of logarithmic growth (data not shown). Vacuoles were visualized by adding 5-chloromethyl fluorescein diacetate (CMFDA; Molecular Probes) at a final concentration of 125 nM in growth media. MitoTracker Red 580 (Molecular Probes) was used at a final concentration of 100 nM in the growth media. Cells were imaged using the appropriate filters on a Zeiss Axiovert M200 microscope.

X-ray Tomography

Stationary phase *S. pombe* were pelleted at $3,000 \times g$ and re-suspended in PBS before being transferred to capillary sample tubes. The cells were then rapidly frozen in a cryogenic gas stream prior to tomographic data collection (see (Le Gros et al., 2005) for a description of the sample tubes and cryogenic sample stage).

Projection images were collected using a transmission soft x-ray microscope (XM-1; beamline 6.1.2 at the Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA). The microscope was equipped with Fresnel zone plate condenser and objective (with 55 nm and 45 nm outer zone widths respectively; the latter being the resolution-defining optical element). The data were collected using x-rays with an energy of 517 eV (2.4 nm), and 16-bit images were recorded using a Peltier-cooled, back-illuminated, 1024×1024 soft x-ray CCD

camera (Roper Scientific Instruments Micromax system with SIT chip; Roper Industries Inc., Duluth, GA). Projection images were collected using exposure times ranging from 0.25 to 1.5 seconds. A full tomographic data set consisted of 90 images collected at 2 degree increments over 180 degrees of rotation. In addition to the data images, 10–12 background images were collected (with the sample moved out of the field of view). Each data image was divided by the average of the background images, and the negative logarithm of the quotient calculated to give images whose gray values correlated directly with the x-ray absorption coefficients.

We assume that the images we collect are good approximations to projections of the absorption coefficients of our sample. This means we neglect any phase effects due to the limited aperture of the optics. Since XM-1 uses a bend magnet x-ray source and uses a Fresnel zone plate condenser, we believe that our assumption of an incoherent source is valid.

As collected, each pixel in the projection image has a width of 10 nm. Prior to alignment and calculation of the tomographic volumes, the images were down-sampled to 512×512 , and therefore a pixel corresponded to 20 nm (approximately half the spatial resolution).

The projection image series were aligned using fiducial markers (60 nm gold particles) with the IMOD software package (Mastronarde, 1997). Reconstruction was carried out using the “Algebraic Reconstruction Technique with blobs” in the XMIPP software package (Marabini et al., 1998; Sorzano et al., 2004). “ART with blobs” is more computationally intensive than the more commonly used filtered backprojection method. However, by using a 20-node cluster of Apple X-serve computers each with two dual-core Intel Xeon processors the reconstructions are calculated in under one hour. After reconstruction, the images were segmented manually, measured, and visualized with Amira (Mercury Computer Systems).

3. Results

For the reconstructed 3-Dimensional volumes from soft x-ray tomography, we used two methods to visualize organelles. The most basic method is a grayscale image of a one-voxel-thick slice through the volume, with the gray value of each voxel corresponding to the soft x-ray absorption coefficient of the material. Figures 1a–c show this type of visualization for four cells (including in a mother and daughter that have not yet separated, Fig. 1c). In Figures 1d–u we display the boundaries between structures that have been digitally segmented from each other. In addition to the plasma membrane of the cells, these images show the borders of organelles within the cell.

(Supplementary Movies 1–3 are available online. In them the cells in Figures 1d–u rotate, and the different organelles are shown one color at a time, to better indicate the distribution of organelles.)

In total, we segmented 50–100 organelles in each cell. We included only organelles with diameters of at least 100 nm, approximately twice the observed spatial resolution in the reconstructions. Table 1 lists the dimensions and volumes of the organelles and cells shown in Figure 1. The cells varied from 4.0 to 6.6 μm long, with volumes of 17 to 31 μm^3 . Despite this variation in size, the volume of the nucleus in these cells appeared to comprise between 4 and 6% of the total cellular volume, and the cumulative volume of other segmented organelles was between 14 and 16% of the total cellular volume. Many of these statistics were based on boundaries that were hand-drawn around the organelles. We subsequently used digital segmentation (boundary drawing) of some of the features multiple times to determine the accuracy with which the boundaries had been drawn, and found that the relative standard deviation was less than 5%.

As mentioned in the Materials and Methods section, by collecting images with the sample in an out of the field, we quantitatively measure projections of the absorption coefficients of our sample, and can then determine these three dimensional absorption coefficients. The values of the voxels in our reconstructed volume thus have value not only because of their contrast with respect to each other, but also because of their absolute values. Every material has a characteristic absorption coefficient (for example, protein, lipid, carbohydrate, water, or glass), so the measured x-ray absorption coefficient can aid in the identification of organelles by giving some indication of their composition. We determined the average x-ray absorption coefficient of each organelle and divided them into five categories, corresponding to the x-ray absorption coefficients indicated in the figure legend (from least to most x-ray dense, the colors are black, blue, green, yellow, and red). The nuclei, which would have an average x-ray absorption coefficient corresponding to blue on the same color scale, are shown in orange to differentiate them from other organelles. Figures 1d–f show all of the color-coded organelles, while figures 1g–u show the five groups of colored organelles in separate panels so that the distributions can be more easily seen. On each row there is also an image of a cross-sectional slice of an organelle characteristic of that group.

Light microscopy was performed for comparison with soft x-ray tomography images. Figure 2 shows these images of *S. pombe* under two different conditions. Figures 2a–d show bright field (a), fluorescence (b, c), and overlay (d) images of log phase yeast in media, where the mitochondria have been labeled with MitoTracker (b) and the vacuoles have been labeled with CMFDA (c). These images show a mitochondrial network that is interconnected through the cell, and show a number of spherical vacuoles in each cell. Figure 2e–h show the corresponding series of images for yeast in PBS which have entered stationary phase. As described in the introduction, in stationary phase yeast there is a cessation of mitochondrial fission, resulting in unbalanced fusion and the formation of a number of spherical mitochondria. Because the cells are in PBS rather than water, thus maintaining the osmolarity of the environment, the vacuole size and number remain approximately the same in these cells.

Our initial assignment of the organelles seen in the x-ray tomography images was carried out on the basis of the organelle appearance and the soft x-ray linear absorption coefficient of the segmented objects. Mitochondria can be readily identified in the tomograms by their characteristic appearance. Whereas most of the organelles have relatively homogeneous absorption coefficients, mitochondria have a thick outer layer that is highly absorptive, while the interior has much lower absorption coefficients, and is very heterogeneous. A cross-sectional image of a mitochondrion and another organelle are displayed in Figures 3a and e, respectively, to illustrate the differences between them. We believe the dark border of the organelle in Figure 3a is the mitochondrial double lipid membrane layers, and the highly heterogeneous interior is due to the cristae. Figures 3b–d show surface visualizations of the mitochondria in each cell (the mitochondria are shown in red).

We identified between 5 and 14 putative mitochondria in each cell, and in each case these mitochondria were distributed throughout the cell and accounted for between 4 and 8% of the cell volume (see Table 1). Because of their heterogeneity, the average absorption of the various mitochondria corresponded to multiple different colors in the scheme of Figure 1. To show the correspondence between the organelles in Figure 1 and Figure 3, matching arrows and wedges have been added to these figures, as described in the figure captions.

4. Discussion

In this work we examined the organelle structure, composition, and distribution in stationary phase *S. pombe* cells using soft x-ray tomography. As presented in the Results section, we determined the sizes and volumes of cells and their organelles at different stages of the cell

cycle, and in particular we analyze the mitochondria in each cell. In a comparable analysis of a single *S. pombe* cell that had been growing in the log phase, the cell was 6.7 μm long and had a volume of 33.5 μm^3 , of which the nucleus comprised 3.16 μm^3 (or 9% of the cell volume) and the mitochondria—which formed a linked network—comprised 1.23 μm^3 (3%) (Hoog et al., 2007).

The differences between the mitochondria in that study and ours are likely based on the different phases in which the yeast were measured. These yeast cells we measured by soft x-ray tomography (as shown in Figure 1 and Figure 3) were placed in PBS for imaging, and thus correspond to the light microscopy images in Figures 2e–h, while the study mentioned in the previous paragraph used yeast comparable to that shown by light microscopy in Figures 2a–d. In another recent report, electron tomography was used to study the transformation of mitochondria during apoptosis (Sun et al., 2007). They found that the cristae matrix fragments and becomes a small number of swollen compartments. Mitochondria of yeast in the stationary phase share some characteristics of mitochondria in cells undergoing apoptosis (Hales, 2004).

We cannot make definitive assignments for the remaining non-mitochondrial organelles without performing further experiments. The surfaces of the segmented non-mitochondrial organelles are shown in Figures 3f–h, along with a characteristic cross section of one of these organelles in Figure 3e. This figure shows that the distribution and number of these remaining organelles is in good agreement with the fluorescence images in 2c and g, in which the vacuoles are labeled. Thus, we believe that a large percentage of these remaining organelles are vacuoles.

(Supplementary Movies 4–6 are available online. In them the cells in Figures 3a–b rotate, and the mitochondria and non-mitochondrial organelles are shown separately, to better indicate their distributions.)

Based on the measured x-ray absorption coefficients of each organelle, we can also determine the likely contents of many of these organelles. For example, lipids have a characteristic linear absorption coefficient that is higher than that of proteins (Henke et al., 1993; Weiss et al., 2000). Thus, the organelles with the highest x-ray absorption coefficients—colored red in Figures 1d–f and shown isolated from other organelles in Figures 1g–i—are most likely filled with lipids.

In comparison with other microscopy techniques, soft x-ray tomography has several advantages for studying the size, distribution, and density of organelles. New ‘super-resolution’ techniques such as stimulated emission depletion microscopy (STED) (Hell, 2007), saturated structured illumination microscopy (SSIM) (Gustafsson, 2005), stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006), photoactivated localization microscopy (PALM) (Betzig et al., 2006), and 4PI confocal microscopy (Egner et al., 2002), can match the resolution of soft x-ray tomography in two dimensions, but in the third dimension they have significantly lower spatial resolutions (~ 100 nm), or are limited to thin sections. In addition, these techniques are limited to information from the fluorescent labels, and do not have access to full structural information on the cell. Electron tomography has been used to analyze organelles in 3-Dimensions at high resolution (Lucic et al., 2005), but in all cases these cells have been chemically fixed, dehydrated, embedded in plastic, and sectioned. Soft x-ray tomography overcomes many of the limitations inherent to the above techniques.

In future work, we will identify organelles through correlative fluorescence and soft x-ray microscopy, using labeling moieties as our guide (Alivisatos et al., 2005). Some organelles clearly have characteristic x-ray absorption profiles, such as the lipid droplets and mitochondria shown here, and we expect that other structures will also show characteristic x-ray absorption profiles.

Soft x-ray tomography is an ideal tool for studying yeast as they respond to environmental changes, especially when correlated with images obtained by light microscopy, such as those in Figure 2. The imaging described in this paper was carried out using a multi-purpose soft x-ray microscope while a new instrument specifically designed for biological and biomedical imaging is being constructed at the Advanced Light Source of Lawrence Berkeley National Laboratory. This new microscope, designated XM2, will produce images with higher spatial resolution and greatly improved signal to noise. With this new microscope we anticipate being able to confidently visualize smaller features with subtle x-ray absorbance variations, for example the microtubule cytoskeleton and territories in the nucleus. This microscope is near completion, and we will use it to continue this investigation of yeast ultrastructure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cells imaged by soft x-ray tomography. **a–c**: One-voxel-thick slices through the reconstructed volumes of four cells (including mother and daughter cells which have not separated), with the gray value of each voxel corresponding to the x-ray absorption coefficient of the material (darker gray = higher absorption). **d–f**: Colored surfaces representing the boundaries of the organelles and the plasma membrane. The colors correspond to the average x-ray absorption coefficients inside the organelles. The scale is shown at the bottom of the figure. The nuclei are colored orange to distinguish them, but have an average x-ray absorption coefficient corresponding to blue. The cell in **a** and **d** is 5 microns in length. **g–u**: The same surfaces as shown in d–f, but with each color shown isolated from the others. On each row in this section,

there is also a cross-sectional image of an organelle characteristic of those displayed in that row. The arrows point to mitochondria, and the wedges point to other organelles. There are corresponding marks on Figure 3, and these are intended to aid in viewing the correspondence between the figures.

Figure 2. Bright field, fluorescence, and overlay images of log phase yeast in media (**a-d**) and stationary phase yeast in PBS (**e-h**). The mitochondria were fluorescently labeled with MitoTracker (**b, f**), and the vacuoles were labeled with CMFDA (**c, g**). Scale bar is 10 microns.

Figure 3.

The same cells as shown in Figure 1, but in **b–d**, only the nucleus and organelles identified as mitochondria are shown (compare to Figure 2**f**), along with a mitochondrial cross section. The remaining organelles are shown in **f–h**, along with a characteristic cross section in **e**. Many of the remaining organelles are vacuoles (compare to Figure 2**g**). The arrows and wedges are described in the Figure 1 caption.

Table 1

Dimensions and volumes of selected organelles and cells from Figure 1.

Feature ^a	Cell			
	early	late	mother	daughter
Cell Length	5.0	6.6	4.5	4.0
Cell Width	2.5	2.8	3.0	2.5
Cell Volume	25	31	25	17
Vol. Nucleus	1.0	1.3	1.1	0.9
# Organelles	71	94	87	57
Vol. Organelles	4.2	4.7	3.9	2.4
# Mitochondria	14	5	11	9
Vol. Mitochondria	1.9	1.4	1.7	0.8

^aLengths are in μm , volumes are in μm^3 .