Soft X-ray Tomography

National Center for X-ray Tomography
Soft X-ray Microscope

- Light source: synchrotron radiation - 2.4 nm $\lambda$, 517 eV
- Optics: zone plates (nano-fabricated nickel Fresnel lenses)
- Contrast mechanism: X-ray absorption by cellular components
• Condenser zone plate focuses source onto specimen
• Objective zone plate magnifies object onto CCD camera

References:
Zone Plate Lenses - Diffractive Optics

• Resolution determined by width of outermost zone of the lens
• As resolution of zone plate increases, depth of focus decreases

Specs of this specific lens:
Diameter = 1 cm
No. of zones = 41,700
Outer zone width = 50 nm
Central stop diameter = 5 mm

Specs of this specific lens:
Diameter = 63 µm
No. of zones = 628
Outer zone width = 25 nm
Nickel plating
Imaging in the Water Window - Absorption Contrast

Between K shell absorption edges of Carbon (284 eV, 4.4nm) and Oxygen (543 eV, 2.3nm)

XM-2 operates at:
517 eV
2.4 nm $\lambda$

Contrast of cell structures is generated by the concentration of organic material (C- and N- containing biomolecules) in each voxel (3D pixel)
Imaging in the Water Window - Absorption Contrast

Structures with many carbon molecules per voxel, such as lipids, have high contrast
X-ray Tomography is Quantitative

Absorption adheres to Beer-Lambert’s law; is linear with thickness, composition & concentration

Absorption coefficient measurements

Absorption coefficient measurements

BSA and hemoglobin

Alcohol oxidase crystal in yeast

Calculated LAC - 0.625 µm⁻¹

Measured LAC - 0.626 ± 0.02 µm⁻¹

Le Gros et al. Cell Reports. (2016) 17(8), 2125-2136

Hemoglobin concentration validated spectrophotometrically

Soft X-ray Tomography

- Whole, hydrated cells in near-native state (cryo-immobilized)
- Natural, quantitative contrast
Tomographic Reconstruction Methods

Types of reconstruction methods:

- **Filtered back projection (FBP)**
  - Noisy
  - Fast

- **Conjugate Gradient Least Squares**
  - Good

- **Penalized-Likelihood**
  - Good

- **L1 regularized Conjugate Gradient Least Squares**
  - Over smoothed
  - Slow

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National Center for X-ray Tomography (NCXT), supported by NIH-NIGMS and DOE-BER
Tomographic Reconstruction Methods

- Penalized ML
- L1-TV modest
- L1-TV high
Measuring Radiation Damage in Full-rotation SXT
Radiation damage - visual inspection

SXT Reconstructions

No visible radiation damage after typical radiation dose

Visible radiation damage after 3x the typical dose

- Red arrowhead: region where the glass capillary has stretched.
- Blue arrowhead: indicates a crack in the specimen.
Radiation damage - quantification

Orthoslices from two fields of view, color-coded according to absorbed radiation dose (see key). Capillary walls absorb highest dose.
Radiation damage - quantification

Orthoslices through a reconstruction of a cell

Graphs of calculated absorbed dose at points on a line (red) drawn through the orthoslices

Capillary walls
Carbon-dense organelle
Radiation damage - inspection with TEM

Vaccinia infected PtK2 cells

Cells on grids imaged with TEM:

Freeze substitute, embedded in Lowicryl, thin section.  

(a, b) Not previously imaged with soft x-ray tomography.  

(c, d) Imaged with TEM after two x-ray tomograms.

Chichon et al., J. Structural Biology (2012) 177:202-211
Measuring Resolution

Full-rotation Tomography
Measuring resolution of soft x-ray microscope

SXM images of test objects

- **25 nm ZP**: 19.5 nm half period
- **15 nm ZP**: 15.1 nm half period

Modulation transfer functions

Resolution Measurement of Yeast Cell

Full rotation tomography with 50 nm resolution zone plate on XM-2

Comparison of Fourier Ring Correlation (FRC) curves calculated with the leave-one-out method.

*S. cerevisiae*

Resolution Measurement of Mammalian Cell

60 nm resolution zone plate

CGLS: Res [nm]: 71.4
L1-TV low: Res [nm]: 60.7
L1-TV modest: Res [nm]: 58.3

In Plane

Transverse

National Center for X-ray Tomography (NCXT), supported by NIH-NIGMS and DOE-BER
Resolution Measurement of Mouse Sperm

Full rotation tomography with 50 nm resolution zone plate on XM-2

Rayleigh resolution measured to be 61 nm (50 nm zone plate)

Mouse sperm
Measuring Resolution

Limited-tilt Tomography
Full Rotation vs. Limited-Tilt Tomography

\[ \pm 90^\circ \]  \quad \pm 72^\circ \quad 16 \, \mu m \text{ thick at } \pm 72^\circ

Full Rotation vs. Limited-tilt Tomography

Doesn’t take into consideration out-of-focus information with increased thickness

180° rotation  
(± 90°)

150° rotation  
(± 75°)

120° rotation  
(± 60°)

90° rotation  
(± 45°)
Measuring Resolution

Limited-tilt tomography using 25 nm resolution zone plate

FRC Criterion: Unlike the Rayleigh criterion which is calculated from high contrast features in an xy slice, the FRC criterion is calculated from the entire tomogram and for all contrast ranges. The resolution achieved at each tilt angle is shown (b). Because this method is sensitive to the thickness of the specimen, the highly tilted images have the worst resolution, while those with lower tilt angles show a best resolution of ~70 nm. While a good comparative measure of the quality of a tomogram, the FRC analysis reflects all the imperfections in the tomographic data, such as the restriction to a limited tilt range, the inclusion of adjacent areas in the tomographic reconstruction, variations in focus due to specimen thickness, and the ever-present noise.

Measuring Resolution

Limited-tilt tomography

4.4 µm thick sample
25 nm zone plate

4.4 µm thick sample
40 nm zone plate

cutoff threshold 0.3

Linear Absorption Coefficient (LAC) Measurements
X-ray Tomography is Quantitative

Absorption adheres to Beer-Lambert’s law; is linear with thickness, composition & concentration

Absorption coefficient measurements


Alcohol oxidase crystal in yeast

Calculated LAC - 0.625 µm\(^{-1}\)
Measured LAC - 0.626 µm\(^{-1}\)

Hemoglobin concentration validated spectrophotometrically
Candida albicans

<table>
<thead>
<tr>
<th></th>
<th>Average LAC (µm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid droplets</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Nucleoli</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>0.20 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 1. Segmentation of organelles based on linear absorption coefficient (LAC) values. (A) A representative diploid cell shown in an orthoslice (i.e. a single slice of tomographic data) and individually segmented organelles; scale bar = 1 µm. (B) LAC values for each organelle. (C) Five different vacuolar compositions found in tomographic data [left; the similar sizes of vacuoles were selected (i.e., ∼1 µm)], schematic views (middle) and LAC values (right; * indicates LAC values of structures inside vacuoles). 0.33, 0.22 and 0.36 µm–1, respectively. Assignment of organelle type to a particular segmented volume was guided by morphological characteristics established by other modalities. For example, the nuclei/nucleoli, mitochondria and vacuoles have distinct and very recognizable morphologies. Once vacuoles from a number of cells had been segmented, it was clear that they could be categorized into one of five types, based on their morphology, internal structure and densities. The LAC values for these are shown in Figure 1C. Representative cells from each stage of the cell cycle are shown in Figure 2A. All the segmented cells were categorized into the appropriate phase of the cell cycle, based on the morphological state of cells and their organelles. It was assumed that these cells were actively going through the cell cycle until the instant they were cryo-immobilized. Using these segmented cells, we quantified cell (Figure 2B), cytosol (Figure 2C) and organelle (Figure 3) volumes, together with their surface areas (Figure 4). In haploid cells, the cell volumes were within the range 10–50 µm³ in G₁, 20–60 µm³ in S, 40–80 µm³ in G₂ and 60–100 µm³ in M phase. In diploid cells, the cell volumes were within 20–60 µm³ in G₁, within 30–80 µm³ in S, within 50–140 µm³ in G₂ and within 70–140 µm³ in M phase. The minimum size requirement to be in G₁ phase was observed to be 10 µm³ in haploid and 20 µm³ in diploid cells. As similar trend to cell volume distribution was also observed in diploid cells of another S. cerevisiae strain (ATCC200060; see supporting information, Figure S1). The average cytosolic volume (Figure 2C) was calculated by combining the cell wall/membrane volume with the total volume.
### Schizosaccharomyces pombe

<table>
<thead>
<tr>
<th></th>
<th>Volume (µm³)</th>
<th>Average LAC (µm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth medium</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>Model protein (C₉₄H₁₃₉N₂₄O₃₁)</td>
<td></td>
<td>1.35</td>
</tr>
<tr>
<td>Glass capillary</td>
<td></td>
<td>1.0</td>
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<tr>
<td>Lipids</td>
<td>0.45</td>
<td>0.72</td>
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<tr>
<td>Mitochondria</td>
<td>2.97</td>
<td>0.42</td>
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<tr>
<td>Nuclei</td>
<td>4.59, 4.90</td>
<td>0.31</td>
</tr>
<tr>
<td>Nucleoli</td>
<td>0.74, 0.71</td>
<td>0.37</td>
</tr>
<tr>
<td>Endosomes</td>
<td>13.9</td>
<td>0.23</td>
</tr>
<tr>
<td>Endosome inclusions</td>
<td>1.15</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Human Lymphoblastoid Cell

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<tr>
<td>Glass capillary</td>
<td>1.0</td>
</tr>
<tr>
<td>Lipid drops</td>
<td>0.73</td>
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<tr>
<td>Mitochondria</td>
<td>0.31 - 0.36</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>0.26 - 0.31</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>0.25 - 0.29</td>
</tr>
<tr>
<td>Heterochromatin</td>
<td>&gt; 0.25</td>
</tr>
<tr>
<td>Euchromatin</td>
<td>&lt; 0.25</td>
</tr>
</tbody>
</table>
3D Volumes of Organelles

Manual Segmentation
Saccharomyces cerevisiae

Diploid

Haploid

- Nucleus
- Nucleolus
- Mitochondria
- Vacuole
- Lipid

Tutorials at ncxt.lbl.gov

Segmentation (using brush tool)

- Highlight a cell region
  - Outline structure with brush
  - Ctrl F to fill
  - Ctrl I to interpolate
- Select label to add that highlighted region; click (+)
- Segment an organelle in all axes (xy, yz, yz)

Slide from segmentation tutorial
3D Volumes of Organelles

Semi-Automatic Segmentation
Segment using Linear Absorption Coefficient

- Manually segment nucleus (red)
- Plot histogram
- High LAC peak corresponds to region of nucleus called heterochromatin (in program Amira)
Segment using Linear Absorption Coefficient

Nucleus seen in Amira

Euchromatin

Heterochromatin

Cytoplasm

Growth medium

Volume [μm^3]

Linear absorption coefficient LAC [1/μm]
Segment using Linear Absorption Coefficient

Mitochondria
0.33 ± µm\(^{-1}\)

Euchromatin
Heterochromatin
Cytoplasm
Nucleus
Growth medium

Linear absorption coefficient LAC [1/µm]
Segment using LAC and manual assist

- Cytoplasm
- Heterochromatin
- Euchromatin / nucleoplasm
- Mitochondria
- Golgi apparatus
- Endoplasmic reticulum
Comparing Soft X-ray Tomography (SXT) and Transmission Electron Microscopy (TEM)
SXT and TEM Comparisons

Vaccinia-infected PtK2 cells

Filaments

Nucleus

Mitochondria

ER

SXT cryo

TEM freeze-sub, Lowicryl

TEM chem. fix, epoxy

Chichon et al., J. Structural Biology (2012) 177:202-211
SXT and TEM Comparisons

Lysosomes

Golgi apparatus

Endoplasmic reticulum

Müller et al. J. Structural Biology. (2012) 177, 179-192
SXT and TEM Comparisons

Filaments

Müller et al. J. Structural Biology. (2012) 177, 179-192
SXT and TEM Comparisons

Microvilli & Plasma membrane

Müller et al. J. Structural Biology. (2012) 177, 179-192
SXT and TEM Comparisons

Nuclear Envelope

Mitochondria

Müller et al. J. Structural Biology. (2012) 177, 179-192

National Center for X-ray Tomography (NCXT), supported by NIH-NIGMS and DOE-BER
SXT and TEM Comparisons

Nucleus

<table>
<thead>
<tr>
<th></th>
<th>EM</th>
<th>SXT Orthoslice</th>
<th>SXT 3D reconstruction</th>
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<tbody>
<tr>
<td>ESC</td>
<td><img src="image" alt="ESC EM" /></td>
<td><img src="image" alt="ESC SXT Orthoslice" /></td>
<td><img src="image" alt="ESC SXT 3D reconstruction" /></td>
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</tr>
<tr>
<td>B</td>
<td><img src="image" alt="B EM" /></td>
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SXT and TEM Comparisons

Nucleus

**Full-rotation tomography, NCXT**

- **SXT**
  - Olfactory epithelial sensory neuron from mouse tissue
  - Mouse lymphoblastoid cell

**Limited-tilt tomography, X-ray Microscope in Berlin**

- **SXT**
  - Mouse adenocarcinoma cells cultured on grids

National Center for X-ray Tomography (NCXT), supported by NIH-NIGMS and DOE-BER
SXT and TEM Comparisons

Nucleoid Organization in *E. coli*

E. Coli hupA38-42 mutant

Thin-section transmission electron photomicrographs illustrating the nucleoid ultrastructure in hupA mutant

Michal Hammel, LBNL
John Tainer, MD Anderson Cancer Center & LBNL
SXT and TEM Comparisons

Autophagic vacuole

Cryo x-ray tomography (A-F)

High pressure freeze, freeze substitute (G-I)

Chen et al. Scientific Reports. (2016) DOI:10.1038/srep34879.
Plasmodium falciparum

**High pressure freeze, freeze substitute**

**Cryo electron tomography**

**Cryo x-ray tomography**

### Table 1. List of electron microscopy fixation methods

<table>
<thead>
<tr>
<th>Fixation method</th>
<th>Abbreviation</th>
<th>Visualization method</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde/reduced osmium tetroxide/thiocarbonyldrazide/OsO₄</td>
<td>ROTO</td>
<td>TEM and electron tomography of serial sections</td>
<td>Good overall preservation of merozoite shape and organelles. Allows visualization of membranes – membrane contrast far superior to standard osmium treatment, but with loss of contrast for proteinaceous structure and DNA. Unsuitable for post-embedding immunolabelling.</td>
<td>Seligman et al. (1966); Willingham and Rutherford (1984)</td>
</tr>
<tr>
<td>High-pressure frozen and freeze substituted</td>
<td>HPF/FS</td>
<td>TEM and electron tomography of serial sections</td>
<td>Methodologically more complex and fixation requires more expensive infrastructure. Excellent preservation of merozoite and organelle structure and shape, lacking ruffles sometimes observed in the chemical fixations (ROTO, GO, GF). Dense granules and mitochondria appeared to be smoother, denser and more turgid. Compatible with post-embedding immunolabelling.</td>
<td>Studer et al. (2008); Waller et al. (2000)</td>
</tr>
<tr>
<td>Cryo-preservation by plunge freezing in liquid ethane</td>
<td>CET</td>
<td>Electron tomography of whole cells (individual)</td>
<td>Excellent whole-cell preservation down to potentially molecular detail. Resolves some cytoskeletal elements not discernible in embedded cells. The resolution and contrast degrade with sample thickness, practically limiting the cell thickness to ~0.5–1 μm. No need for staining. Not readily suitable for immunolabelling internal structures</td>
<td>Cyrklaff et al. (2007); Kudryashev et al. (2010)</td>
</tr>
</tbody>
</table>

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Mitochondria changes in mast cell degranulation

Segmented images of cells from SXT reconstructions before (A) and after (C) 30 min of activation. (B,D–F) 3D reconstructed structures from SXT in the rectangle area of (A) and I, II, III of (C).

Chen et al. Scientific Reports. (2016) DOI:10.1038/srep34879.
Structures Imaged Using SXT

Endosomes

Duke et al.
Ultramicroscopy
(2014) 143, 77-87
Comparisons between X-ray Tomography & Light Microscopy
Mitochondria in the Yeast, *S. cerevisiae*

Wild type  
dnm1 knockout  
fzo1 knockout

Mitotracker
Membrane Invaginations in Yeast, *S. cerevisiae*

FM-64 live-cell stain

Wild type

sjl1Δ sjl2Δ mutant

SXT of sjl1Δ sjl2Δ synaptojanin mutant

Candida albicans

Yeast-like

Hyphal

Peptoid 1-treated

Peptoid 2-treated

Mouse Olfactory Epithelial Cells

Multipotent stem cell

Neuronal progenitor

Mature neuron

Silenced Genes in Olfactory Sensory Neuron

Nucleoid Organization in *E. coli*

Super-resolution imaging of major nucleoid-associated proteins in living *E. coli* cells.

Michal Hammel, LBNL
John Tainer, MD Anderson Cancer Center & LBNL
Correlated Fluorescence and X-ray Tomography - Same Cell
Correlated Fluorescence and X-ray Tomography


National Center for X-ray Tomography (NCXT), supported by NIH-NIGMS and DOE-BER
Vacuoles in *S. pombe*

**Fluorescence**

**Soft X-ray Tomography**

Lipid drops labeled with BODIPY

Lipid drops easily identified in SXT because they are very highly absorbing

Lysosomes Labelled with Lysotracker

Nucleus and Nucleolus in Yeast, *S. cerevisiae*

Walters et al. Current Biology. (2014) 24(23), 2861-2867
Inactive X chromosome

2D orthoslices from fluorescence tomography (MacroH2A-EGFP)

2D orthoslices from soft x-ray tomography

Overlay of above fluorescence on SXT orthoslices

Inactive X chromosome

ER-Mitochondria Contact Sites

ER-Mitochondria Contact Sites

Over expression of MiD51 results in fragmented mitochondria that have a decreased LAC value (are less x-ray absorbing).

![Mitochondria and Endoplasmic reticulum](image)

Over expression of MiD51 results in fragmented mitochondria that have a decreased LAC value (are less x-ray absorbing).

![Graph showing relative LAC values](image)

Nucleolus

FISH

SXT Orthoslice

LAC, 0.19 μm⁻¹

LAC, 0.23 μm⁻¹

LAC, 0.27 μm⁻¹