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Tomographic imaging of biological specimens with the cryo transmission X-ray microscope

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Abstract

Using the photoelectric absorption contrast between water and protein at 2.4 nm wavelength, cryo X-ray microscopy has visualized protein structures down to 30 nm size in unstained, unsectioned biological specimens. Due to the large depth of focus of the Fresnel zone plate objectives, computed tomography based on a tilt series of X-ray microscopic images can be used to reconstruct the three-dimensional specimen structure. This method has been applied to the green alga *Chlamydomonas reinhardtii*, and to cell nuclei of male *Drosophila melanogaster* fruit fly cells.

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1. X-ray microscopy

Using radiation from the 'water window' wavelength range (2.34–4.38 nm, [1]), the intrinsic absorption contrast between protein and water can be used to image unstained hydrated biological specimens of up to 10 μm thickness. X-ray microscopy with Fresnel zone plate objectives has visualized 30 nm frozen-hydrated organic structures in cells [2]. To image low-contrast object details with sufficient statistical significance in absorption contrast mode, the specimens have to be exposed to radiation doses of approximately 10^7 Gy. If multiple images are acquired (such as for computed tomography), the specimens are exposed to a cumulative dose of up to 10^9 Gy. Imaging the specimens at a cryogenic temperature of approximately 110 K prevents radiation damage at the microscopic resolution for doses of up to 10^{10} Gy [3]. The large depth of focus of the Fresnel zone plate objectives permits to treat the X-ray microscopic images in good approximation as projections of the specimen absorption [4]. Computed tomography (CT) can therefore be used to reconstruct the local linear absorption coefficient from a tilt series of images acquired

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under different viewing angles. This has already been demonstrated with micro-fabricated test patterns [5], the mineral sheaths of bacteria [6], and frozen-hydrated mouse 3T3 fibroblasts [7]. In this work, CT is used to reconstruct cryogenic biological specimens of *Chlamydomonas reinhardtii* and cell nuclei of *Drosophila melanogaster*, and the resolution of the reconstructions is evaluated.

2. Investigated specimens

All experiments described in this work were performed with the cryo transmission X-ray microscope at the electron storage ring BESSY I [2]. CT was used to investigate the unicellular green alga *Chlamydomonas reinhardtii* (SAG classification 11-32b), which has been previously studied with X-ray microscopes [8,2]. Live specimens were inserted into a capillary holder and frozen by plunging the holder into liquid nitrogen. A suitable specimen was selected in the cryo X-ray microscope, and 42 tilt images were acquired at a temperature of 113 K, spanning an angular range of 185°. The specimen was reconstructed using a multiplicative algebraic technique [9], yielding a three-dimensional (3D) map of the local linear absorption coefficient [4]. However, due to the insufficient cooling properties of liquid nitrogen (Leydenfrost phenomenon), the nucleus and nucleolus were damaged by ice crystal formation during the cooling process (cf. Fig. 1(a)).

Plunge-freezing in liquid ethane has been shown to considerably improve the vitrification of biological specimens for cryo X-ray microscopy [2]. Consequently, the CT experiment was repeated using liquid ethane as the primary cryogen. Fig. 1 shows virtual sections through a liquid-ethane frozen specimen in panels (b)–(f), obtained by slicing the reconstructed three-dimensional volume in parallel planes of 0.34 μm separation. The section thickness is equal to the voxel size (26 nm). The panels are thus similar to a focus series acquired with a very small depth of focus.

By comparing panel (a) to panels (b)–(f), it can be seen that the preservation of the specimen structure is significantly improved, with the nucleus and nucleolus clearly visible. Whereas for the liquid-nitrogen frozen specimen much of the specimen interior has the same linear absorption coefficient as the surrounding water ($\approx 0.1\ \mu m^{-1}$, panel (a)), for the liquid-ethane frozen specimen only the vacuole has the same linear absorption coefficient as water (panel (f)), and the cytosol surrounding the organelles is characterized by a homogeneous absorption coefficient of approximately $0.25\ \mu m^{-1}$, indicating that on the resolution scale of the X-ray microscope, the biological material has not been segregated by ice crystal formation.

The distribution of the dosage compensation protein MSL-1 in male *Drosophila melanogaster* fruit fly cells (SL2; ATCC number CRL#1963) has been visualized in conventional two-dimensional X-ray micrographs using immunogold labeling and silver enhancement [10]. While the lateral resolution in these micrographs is much better than with confocal laserscan microscopy, little information about the axial distribution of the protein can be gained. CT reconstruction was therefore applied to the cell nuclei to map the 3D distribution of the MSL-1 protein. First results indicate that the reconstructed local linear absorption coefficient may also be used advantageously to clearly distinguish between labeled sites and regions which appear labeled due to overlap of protein dense parts situated at different depths in the specimen.

3. Tomographic resolution

The local linear absorption coefficient is reconstructed in planes perpendicular to the axis of object rotation, and the stack of reconstruction planes forms the three-dimensional reconstructed volume. Because the reconstruction planes are independent of each other, the resolution perpendicular to the planes is equal to the resolution of the X-ray microscope (neglecting misalignment of the tilt series). The resolution in the reconstruction planes is usually evaluated by taking into account the nature of the reconstruction process: the Fourier coefficients of the unknown object function are known only for those central sections of

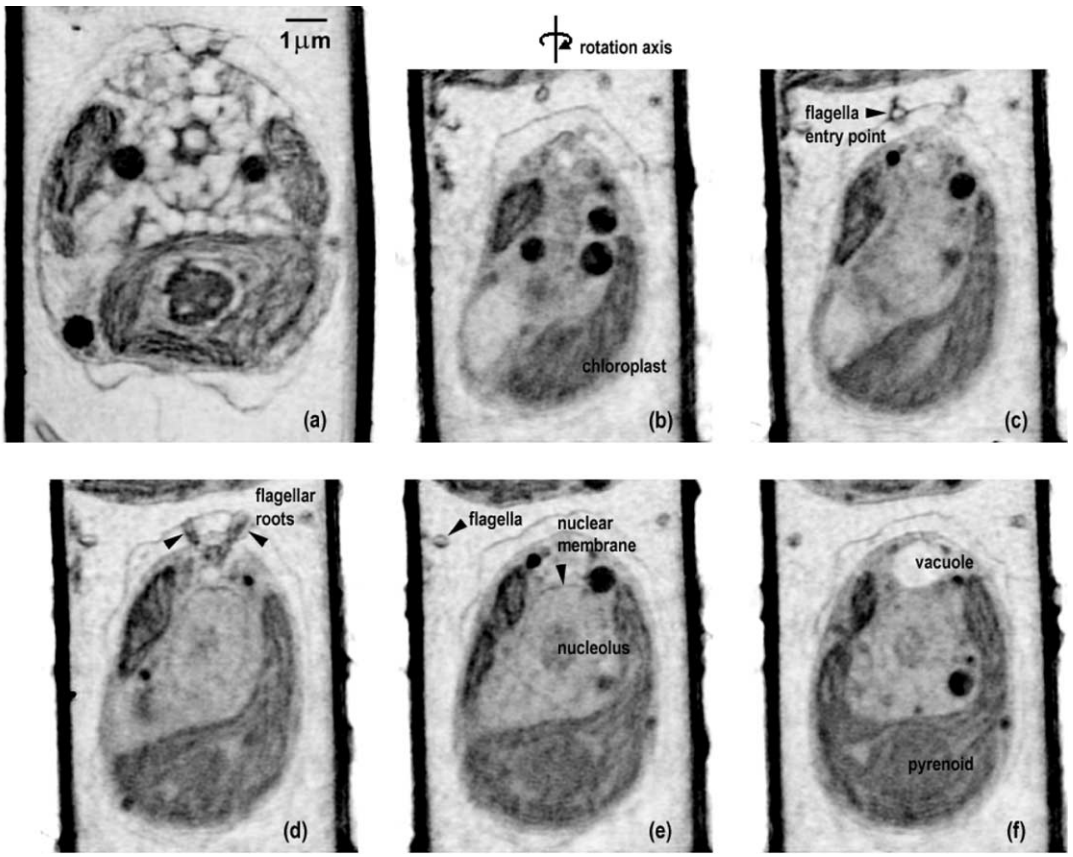

Fig. 1. Virtual sections of *C. reinhardtii*, obtained from reconstructions of the linear absorption coefficient of a specimen frozen in liquid nitrogen (panel (a)) and a specimen frozen in liquid ethane (panels (b)–(f)). The sections are 26 nm thick and parallel to the axis of object rotation. Panels (b)–(f) show parallel planes of 0.34 μm separation. Low absorption maps to light gray, and high absorption to dark gray.

Fourier space that correspond to experimentally acquired projections; in between these central sections, the Fourier components have to be interpolated. The *Crowther resolution* specifies the maximum spatial frequency for which unknown Fourier components can be interpolated consistently, as $k_c = 1/(\Delta\theta\, D)$, where $\Delta\theta$ is the tilt angle increment and D the object diameter [11]. Table 1 shows the specimen diameter and the Crowther resolution for the three reconstructed specimens.

The consistency of the interpolation can also be measured directly. The *differential phase residual* (DPR) compares two independent reconstructions of the same specimen (computed, for example, from the even- and the odd-numbered projections of the tilt series):

$$\Delta\varphi(k,\Delta k) = \left| \frac{\sum_{[k,\Delta k]} (\Delta\varphi(\boldsymbol{k}))^2 (|F_1(\boldsymbol{k})| + |F_2(\boldsymbol{k})|)}{\sum_{[k,\Delta k]} (|F_1(\boldsymbol{k})| + |F_2(\boldsymbol{k})|)} \right|^{1/2} \tag{1}$$

where $\Delta\varphi(\boldsymbol{k}) = \varphi_2(\boldsymbol{k}) - \varphi_1(\boldsymbol{k})$ is the phase difference between the Fourier transforms $F_{1,2}(\boldsymbol{k}) = A_{1,2}(\boldsymbol{k})e^{i\varphi_{1,2}(\boldsymbol{k})}$ of the two reconstruction planes, and the sums extend over annular regions in Fourier space with inner radius k and outer radius $k + \Delta k$. Low values of $\Delta\varphi$ indicate good agreement between the two reconstructions. As k increases, more Fourier components have to be interpolated,

Table 1
Specimen diameter D, Crowther resolution k_c, and average DPR resolution k_{45} (cf. Fig. 2) of the specimens. The Crowther resolution was calculated for a common tilt angle increment $\Delta\theta = 4.52^\circ$

Specimen	D (μm)	k_c (μm^{-1})	k_{45} (μm^{-1})
LN_2 frozen alga	8.7	1.5	2.0
Liquid-ethane frozen alga	7.1	1.8	2.1
D. cell nuclei	8.15	1.6	1.8

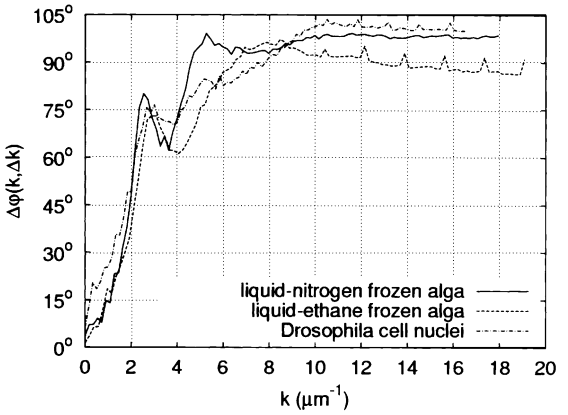

Fig. 2. Differential phase residual (DPR) $\Delta\varphi(k, \Delta k)$ for the reconstructed specimens (averaged over all reconstruction planes), with Δk chosen as 0.01 of the Nyquist frequency. The DPR resolution ($\Delta\varphi = 45^\circ$) is 1.8 μm^{-1} (cell nuclei), 2.0 μm^{-1} (liquid-nitrogen frozen alga), and 2.1 μm^{-1} (liquid-ethane frozen alga).

and the agreement between the two reconstructions is reduced, resulting in larger values of $\Delta\varphi$. A resolution criterion is defined by stipulating that $\Delta\varphi(k_{45}) = 45^\circ$ [12]. Fig. 2 shows the DPR graphs for the three specimens. As can be seen from Table 1, the multiplicative algebraic reconstruction technique achieves a DPR resolution k_{45} that is somewhat better than the corresponding Crowther resolution. However, due to the relatively small number of tilt angles, it is much smaller than the microscopic resolution of approximately 20 μm^{-1}. To achieve a higher Crowther/DPR resolution, the tilt angle increment must be decreased, i.e., more tilt images must be acquired.

4. Summary

CT was used to reconstruct cryogenic hydrated biological specimens from tilt series of X-ray microscopic images. Improved vitrification was achieved using liquid ethane as the primary cryogen. The tomographic resolution in the direction of the rotation axis is approximately equal to the microscopic resolution. While fine specimen details are also visible perpendicular to that axis, the comparison of Crowther resolution and microscopic resolution shows that the number of tilt views should be increased.

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