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Three-dimensional mass density mapping of cellular ultrastructure by ptychographic X-ray nanotomography

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ABSTRACT

We demonstrate absolute quantitative mass density mapping in three dimensions of frozen-hydrated biological matter with an isotropic resolution of 180 nm. As model for a biological system we use *Chlamydomonas* cells in buffer solution confined in a microcapillary. We use ptychographic X-ray computed tomography to image the entire specimen, including the 18 μm-diameter capillary, thereby providing directly an absolute mass density measurement of biological matter with an uncertainty of about 6%. The resulting maps have sufficient contrast to distinguish cells from the surrounding ice and several organelles of different densities inside the cells. Organelles are identified by comparison with a stained, resin-embedded specimen, which can be compared with established transmission electron microscopy results. For some identified organelles, the knowledge of their elemental composition reduces the uncertainty of their mass density measurement down to 1% with values consistent with previous measurements of dry weight concentrations in thin cellular sections by scanning transmission electron microscopy. With prospects of improving the spatial resolution in the near future, we expect that the capability of non-destructive three-dimensional mapping of mass density in biological samples close to their native state becomes a valuable method for measuring the packing of organic matter on the nanoscale.

1. Introduction

The capability of visualizing with high resolution the three-dimensional (3D) structure of biological tissue has provided valuable insights into the cellular ultrastructure (Leis et al., 2009) and extracellular organization, e.g., of neural networks (Helmstaedter et al., 2008). With sufficient resolution to visualize cell membranes, electron microscopy (EM) is frequently used for imaging cellular ultrastructure. Cryo-fixation avoids radiation damage and sample movements during acquisition while keeping the specimen close to its native state (Adrian et al., 1984). Vitrification processes are used in such cases to prevent the creation of ice crystals which destroy the delicate cellular structure. However, the short penetration depth of electrons requires slicing, which can produce artifacts (Al-Amoudi et al., 2005).

Apart from the cellular structure information provided by images, physical quantities such as the mass density allow insight into the organization of molecules in the cellular matrix. This information is typically gained by density gradient centrifugation techniques, which usually require measurements of purified substances in vitro (Yoon and Lim, 2003). Some imaging techniques yield simultaneously spatially resolved structural information with quantitative mass density information. For example, the darkfield contrast in scanning transmission electron microscopy (STEM) of thin slices scales with the mass. This technique is used in combination with X-ray microanalysis to estimate weight concentrations of different substances in cellular compartments while they remain

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in their natural environment (von Zglinicki and Bimmler, 1987). Together with further biochemical and biophysical analysis, resolving in 3D the nanoscale density distribution could provide new valuable insight into molecular packing in cellular and extracellular compartments in context with the cell or tissue in which they are embedded.

Complementary to cryo-EM, cryo X-ray microscopy is capable of resolving cellular ultrastructure down to about 30 nm resolution (Parkinson et al., 2008; Schneider et al., 2010; Chichón et al., 2012; Hanssen et al., 2012). Typically used in absorption contrast at energies within the so-called water window, i.e., the energy range between absorption edges of carbon and oxygen, it provides high contrast between biological matter and water, facilitating 3D visualization of entire cells with thicknesses up to about 10 μm without sectioning. For samples thicker than 10 μm, the X-ray energy must be increased, and phase-sensitive X-ray microscopy concepts such as holo-tomography (Cloetens et al., 1999), Zernike phase contrast (Stampanoni et al., 2010), or coherent diffraction imaging (CDI) (Jiang et al., 2010) need to be used because of the reduced absorption contrast of biological tissues at such energies. Here one measures the phase shift of the X-ray wave field as it propagates through the sample as contrast mechanism instead of the attenuation of the wave field. Phase contrast is mostly related to the mass density variations within the specimen. Therefore, phase tomography offers the possibility to map the specimen's 3D mass density distribution on the nanoscale.

The technique of ptychographic X-ray computed tomography (PXCT) enables 3D density mapping of samples tens of micrometers in size with resolutions down to tens of nanometers (Dierolf et al., 2010). Ptychography is a CDI modality which consists of scanning a specimen across a confined coherent illumination in such a way that illuminated areas partially overlap at various scan positions (Rodenburg et al., 2007). At each scanning step, coherent diffraction patterns are recorded in the far field, and phase retrieval algorithms are used to reconstruct the complex-valued transmissivity of the specimen (Faulkner and Rodenburg, 2004). The combination of phase images acquired at different incident angles of the beam serve for 3D tomographic reconstruction of the specimen. As in CDI, the resolution in PXCT is only limited, in theory, by the largest diffraction angle at which intensities can be reliably detected, but in practice it is often limited by positioning accuracy. Moreover, with PXCT one can arbitrarily extend the field of view of the specimen, which is limited in plane-wave CDI, where the sample needs to be confined to a few micrometers in size due to the combination of a support constraint and a sampling requirement (Miao et al., 1998). Apart from a few reported experiments (Lima et al., 2009; Rodriguez et al., 2015), this limitation has hampered the application of CDI for cryo imaging, where surrounding ice layers are incompatible with a small, confined sample. Ptychographic algorithms with simultaneous reconstruction of the illumination (Guizar-Sicairos and Fienup, 2008; Thibault et al., 2008; Maiden and Rodenburg, 2009) are very robust to reconstruct thick objects producing phase shifts of many waves (da Silva et al., 2015) while keeping a high phase sensitivity of 0.005 rad (Lima et al., 2013). Therefore, the combination of X-ray ptychography with tomographic reconstruction results in high-quality 3D density maps (Diaz et al., 2012) which are increasingly used as a characterization tool in multiple scientific fields (da Silva et al., 2015; Chen et al., 2013; Fløystad et al., 2015; Dam et al., 2014). Using a recently developed instrument with high accuracy positioning, a resolution of 16 nm in 3D has been demonstrated (Holler et al., 2014). Ptychography has been successfully tested for 2D imaging of biological matter, both with freeze-dried (Giewekemeyer et al., 2010; Jones et al., 2014) and frozen-hydrated specimens (Lima et al., 2013; Deng et al., 2015), and for 3D imaging of freeze-dried specimens (Guizar-Sicairos et al., 2011; Wilke et al., 2012; Jones et al., 2013).

Here we demonstrate PXCT at an energy of 6.2 keV for 3D density mapping of frozen-hydrated biological matter. We show 3D maps of intact *Chlamydomonas* cells at 180 nm resolution with quantitative mass density values within 6% accuracy and with sufficient contrast to distinguish several organelles. Comparing with additional measurements on stained resin-embedded *Chlamydomonas* and earlier literature on EM and absorption X-ray microscopy, we identify the cell organelles and determine their mass densities in their hydrated state.

2. Materials and methods

2.1. Sample preparation

Chlamydomonas reinhardtii 137c wild type were obtained from the Chlamydomonas Genetic Center. Cells were cultured in Tris–acetate–phosphate (TAP) medium (Gorman and Levine, 1965). For the frozen-hydrated specimen preparation a quartz glass capillary of 1.20 mm outer diameter and 0.90 mm inner diameter was pulled to obtain a thin tip of about 7 μm diameter and tapering walls. After pulling, the capillary was fixed with glue to a standard 3 mm diameter brass pin from Hampton Research (CA, USA). Cells in their medium with a volume concentration of 8.5% glycerol as cryo-protectant were injected from the wide end of the capillary using a microliter syringe and needle, in such a way that the solution filled the thin part of the capillary up to the top. In Fig 1(b) we show a light microscope image of the confined cell solution in the microcapillary captured immediately before plunge freezing. The brass pin was then mounted in a sample mount with magnetic base from Hampton Research, and plunge frozen in liquid ethane. After plunging, the sample was stored in liquid nitrogen for a few days until the experiment took place.

For the preparation of the resin-embedded specimen, cells in liquid TAP medium were pelleted at 800 g for 8 min. The pellet

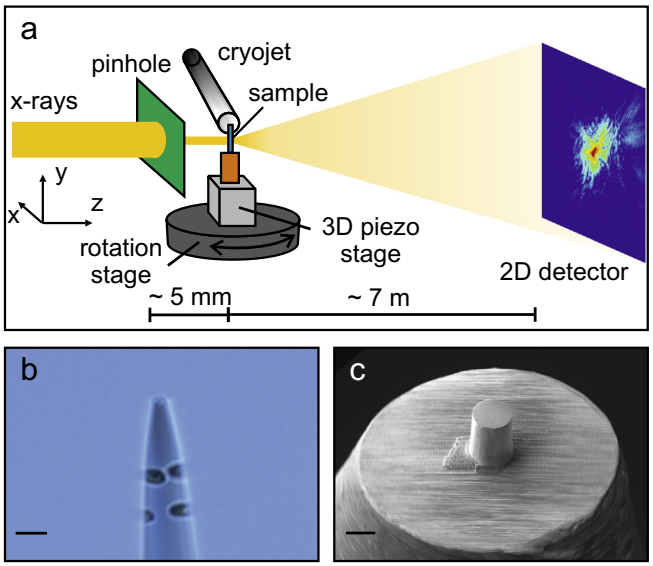

Fig.1. (a) Sketch of the setup used for ptychographic tomography measurements. (b) Light microscope image of *Chlamydomonas* specimen in solution confined in a glass microcapillary right before plunge freezing. (c) SEM image of stained, resin-embedded *Chlamydomonas* specimen prepared by focused ion beam (FIB). Scale bars in (b) and (c) are 20 μm.