Journal of Structural Biology 181 (2013) 77–81

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Technical Note

Vitrification of thick samples for soft X-ray cryo-tomography by high pressure freezing

Allon Weiner [a], Sergey Kapishnikov [a], Eyal Shimoni [b], Sandra Cordes [a], Peter Guttmann [c], Gerd Schneider [c], Michael Elbaum [a,*]

[a] Dept. of Materials and Interfaces, Weizmann Institute of Science, Rehovot 76100, Israel
[b] Chemical Research Support Dept., Weizmann Institute of Science, Rehovot 76100, Israel
[c] Helmholtz Zentrum für Materialien und Energie GmbH, Institute for Soft Matter and Functional Materials, Albert-Einstein-Str. 15, 12489 Berlin, Germany

ARTICLE INFO

Article history:
Received 8 August 2011
Received in revised form 3 September 2012
Accepted 8 October 2012
Available online 16 October 2012

Keywords:
X-ray microscopy
Tomography
Electron cryo-microscopy
High pressure freezing
Water-window
Plasmodium

ABSTRACT

Soft X-ray cryo-microscopy (cryo-XT) offers an ideal complement to electron cryo-microscopy (cryo-EM). Cryo-XT is applicable to samples more than an order of magnitude thicker than cryo-EM, albeit at a more modest resolution of tens of nanometers. Furthermore, the natural contrast obtained in the "water-window" by differential absorption by organic matter vs water yields detailed images of organelles, membranes, protein complexes, and other cellular components. Cryo-XT is thus ideally suited for tomography of eukaryotic cells. The increase in sample thickness places more stringent demands on sample preparation, however. The standard method for cryo-EM, i.e., plunging to a cryogenic fluid such as liquid ethane, is no longer ideally suited to obtain vitrification of thick samples for cryo-XT. High pressure freezing is an alternative approach, most closely associated with freeze-substitution and embedding, or with electron cryo-microscopy of vitreous sections (CEMOVIS). We show here that high pressure freezing can be adapted to soft X-ray tomography of whole vitrified samples, yielding a highly reliable method that avoids crystallization artifacts and potentially offers improved imaging conditions in samples not amenable to plunge-freezing.

1. Introduction

The potential advantages of soft X-ray microscopy have long been recognized: extended penetration depth with respect to electron microscopy due to differences in the interaction of electrons or X-rays with matter, and high resolution compared with conventional visible light microscopy due to the shorter wavelength of X-rays (Kirz et al., 1995; Schneider, 1998). X-ray microscopy is poised to fill an important gap for cellular imaging in particular. On one hand, in the light microscope image contrast modalities based on scattering or phase interference, including dark field, Zernike phase contrast, and differential interference contrast, are limited in resolution by Abbe's constraint, $d = 0.61\lambda/\mathrm{NA}$. Numerically this is on the order of 250 nm for visible light and high numerical aperture objectives. Widefield fluorescence imaging suffers from the same constraint, which originates in the maximal spatial frequency that can be transmitted through the objective. Structured illumination can effectively double this resolution (Gustafsson et al., 2008), and new techniques based on constrained excitation (e.g., STED) or localization of individual fluorophores (e.g., PALM, STORM, or GSDIM) reach resolutions into the tens of nanometers (Toomre and Bewersdorf, 2010). However they can reveal only the labeled molecules and not the surrounding cellular context. In the electron microscope, on the other hand, multiple and inelastic scattering processes reduce image contrast. This effect can become significant for samples as thin as 100 nm. High excitation voltages and zero-loss energy filters can reduce the contribution of inelastic scattering to image formation and extend the sample thickness range to 500 nm or beyond in advantageous cases (Ben-Harush et al., 2010). Damage due to inelastic interactions still limits the total permissible dose of electrons, however. Furthermore, as samples become thicker than the size of objects under view, the images become difficult to interpret without information on relative locations in the third dimension. Thus tomography enters naturally, and here the benefits of X-ray microscopy, i.e., the ability to image much thicker samples than possible by electron microscopy, come to full advantage (Schneider et al., 2002, 2010; Larabell and Nugent, 2010; Hagen et al., 2012; Kapishnikov et al., 2012).

Soft X-ray microscopy in the "water-window" between the K-absorption edges of carbon at 284 eV and oxygen at 543 eV offers an intrinsic and quantitative mechanism of differential contrast between organic and aqueous media. Membrane boundaries of cellular organelles are well delineated without chemical stain. The

* Corresponding author. Fax: +972 8 9344138.
E-mail address: michael.elbaum@weizmann.ac.il (M. Elbaum).

1047-8477/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jsb.2012.10.005

characteristic absorption length of water just before the oxygen K-edge is approximately 10 μm. This is large enough to accommodate many types of eukaryotic cells in their entirety. With the increase in thickness scale from <0.5 to ~10 μm, however, the demands on sample preparation become considerably more stringent. Ideally samples would be vitrified as is done routinely in cryo-EM. Achieving reliable vitrification in such thick samples is not trivial, however, and unwanted formation of ice crystals can induce severe sample damage (Studer et al., 1995).

Two distinct approaches have been taken to vitrify samples for soft X-ray microscopy, depending on the construction of the microscope. In one case, the sample is introduced into a narrow capillary 10 μm in diameter that can be rotated around its long axis to provide tomographic data collection (Schneider et al., 2002; Larabell and Le Gros, 2004; McDermott et al., 2009). Such samples are typically frozen by plunging the capillary into liquid nitrogen, and then maintained at low temperature by means of a helium gas jet pre-cooled in liquid nitrogen. The second approach, currently implemented at the U41 beamline at BESSY II, uses flat grids mounted in a holder designed originally for electron cryo-tomography (Carrascosa et al., 2009; Schneider et al., 2010, 2012). The sample is typically vitrified by plunging the grid rapidly into a cryogenic fluid such as liquid ethane.

Thin aqueous films cool very quickly and homogeneously during plunge-freezing to yield, ideally, a fully vitrified sample (Adrian et al., 1984; Talmon, 1986). Cryo-plunging is considered the state of the art for preparation of protein and virus samples for structure determination by cryo-TEM. It has been extended for microscopy and tomography of selected cellular specimens (Medalia et al., 2002; Beck et al., 2004; Jensen and Briegel, 2007). In general the intrinsic limitations of imaging thick samples by TEM set in before the limitations of vitrification by plunging. Indeed, Dubochet and Lepault state in one of their early papers describing this method: "It is the good fortune of the electron microscopist that, reducing sample size in order to increase the cooling speed, vitrification becomes easy just when the dimensions of the specimen are those suitable for electron microscopy." (Dubochet and Lepault, 1984).

For X-ray imaging this is not the case. Useful imaging signals may be collected even for 15–20 μm sample thickness, and vitrification by the plunging method is then far from easy (see Supplementary material for analysis). For specimens of several μm thickness one cannot expect that the center of the sample will cool at the same rate as the faces. Indeed, in our experience vitrification is no longer reliably obtained. Grains reminiscent of hexagonal ice often appear in the images. Very often an isolated damage plane appears in tomographic reconstruction, occasionally two. With luck these may not overlap the plane of interest in the sample. Not satisfied with this element of luck, we undertook to adapt the method of high pressure freezing to sample preparation for soft X-ray microscopy.

High pressure freezing achieves vitrification by rapid cooling under very high pressure (Shimoni and Muller, 1998). Application of pressure has two effects: (a) thermodynamically, to depress the equilibrium freezing temperature, maximally at 2100 atm to −21.5 °C, and (b) kinetically, to slow the growth of ice crystals should nucleation occur. High pressure freezing is considered the state of the art in preservation of thick specimens for electron microscopy. It may be followed by freeze-fracture for surface

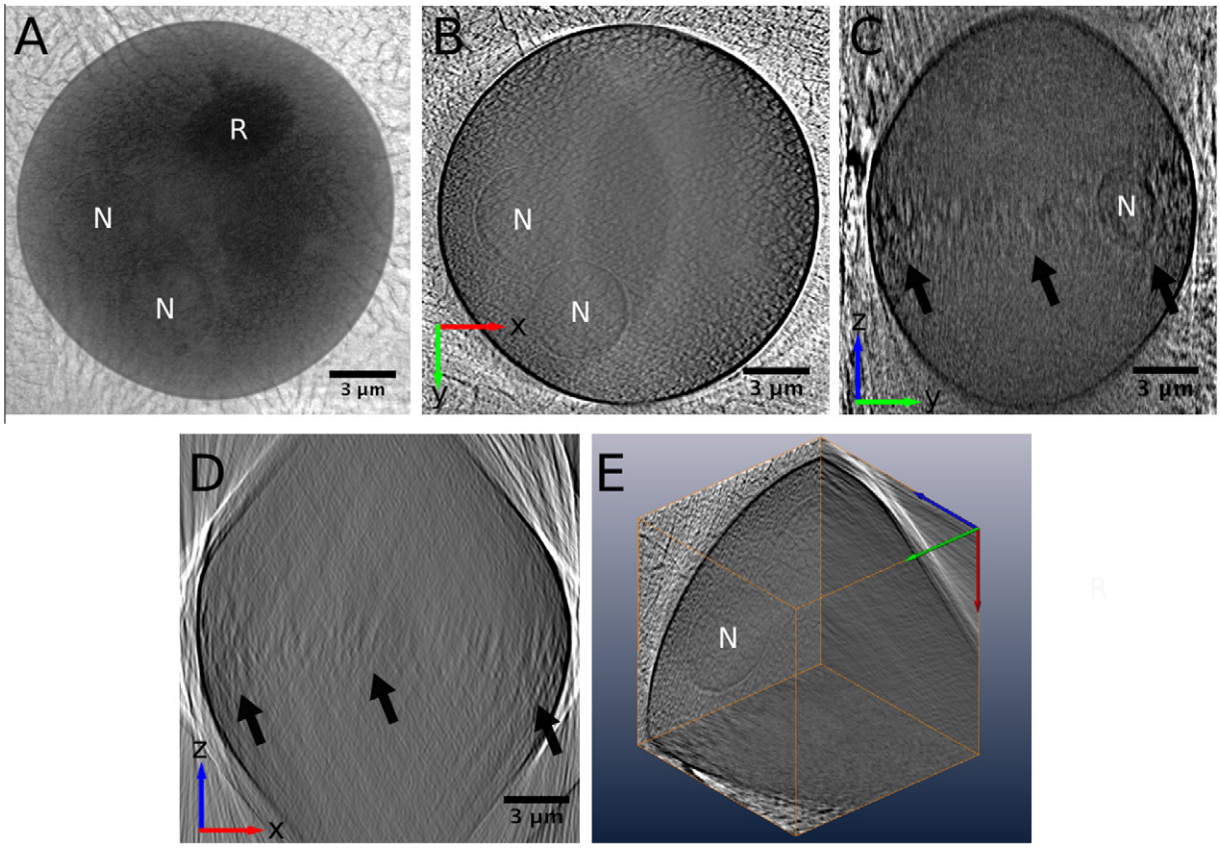

Fig.1. Characteristic crystallization damage in thick samples prepared by plunging to liquid cryogen. A cyst of *Entamoeba invadens* is shown in panels (A–E): (A) zero-tilt projection view, with nuclei (N) and ribosome crystal (R) visible. Contrast was compressed logarithmically in order to show both internal detail and the cracked appearance of the background. (B) A slice through the equatorial plane of the reconstruction, showing two nuclei and the grains of ice. (C,D) Orthogonal views in which the most significant damage is seen (indicated by arrows) restricted to the central plane, with thickness 2–3 μm. (E) A cut-out view through the volume showing the relative position of the damage in three dimensions.