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Cryo-planing of frozen-hydrated samples using cryo triple ion gun milling (*CryoTIGM*™)

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ABSTRACT

Cryo-SEM is a high throughput technique for imaging biological ultrastructure in its most pristine state, i.e. without chemical fixation, embedding, or drying. Freeze fracture is routinely used to prepare internal surfaces for cryo-SEM imaging. However, the propagation of the fracture plane is highly dependent on sample properties, and the resulting surface frequently shows substantial topography, which can complicate image analysis and interpretation. We have developed a broad ion beam milling technique, called cryogenic triple ion gun milling (*CryoTIGM*™ [ˈkrī-ə-ˌtīm]), for cryo-planing frozen-hydrated biological specimens. Comparing sample preparation by *CryoTIGM*™ and freeze fracture in three model systems, Baker's yeast, mouse liver tissue, and whole sea urchin embryos, we find that *CryoTIGM*™ yields very large (~700,000 μm^2) and smooth sections that present ultrastructural details at similar or better quality than freeze-fractured samples. A particular strength of *CryoTIGM*™ is the ability to section samples with hard-soft contrast such as brittle calcite ($CaCO_3$) spicules in the sea urchin embryo.

1. Introduction

Electron optical imaging of biological ultrastructure, in combination with correlative techniques, has made fundamental contributions to our understanding of the hierarchical arrangement and function of living, synthetic, and hybrid systems (Denk and Horstmann, 2004; Fridman et al., 2012; Heymann et al., 2009; Kenzie et al., 2012; Mahamid et al., 2010; Pawley, 2008; Schatten, 2011; Sousa and Leapman, 2012; Studer et al., 2008; Walther, 2003). However, artifacts from sample preparation steps, e.g. chemical fixation and drying, often impact the identification and interpretation of the structural details. Cryo-fixation and imaging of specimens embedded 'frozen-hydrated' in amorphous ice allows their observation in a near-native state. Imaging of cryo-fixed samples by cryo-SEM is particularly attractive due to ease of preparation and the excellent resolution of current instruments.

Conventional sample preparation for TEM involves chemical fixation, followed by removal of water, embedding in a resin, and preparation of thin-sections (Winey et al., 2013). Drawbacks of this approach are that each of these steps is the cause for artifacts (Gilkey and Staehelin, 1986; Hoch, 1991; Studer et al., 2008; Winey et al., 2013), and that highly mobile constituents of the sample, for instance many inorganic ions, are redistributed or lost in the process. Cryo-fixation (Dubochet, 2007) is not only much more rapid than chemical fixation (Hoch, 1991), but preserves samples in a fully-hydrated frozen state, thus providing a snapshot of the distribution of all soluble and insoluble components at the time of vitrification. With the development of high-pressure freezing (HPF), samples several hundred micrometers in thickness can be analyzed without using cryoprotectants to suppress growth of ice crystals (Gilkey and Staehelin, 1986; Meryman, 2007; Moor, 1987; Studer et al., 2001). HPF samples can be dehydrated by freeze substitution (McDonald, 2007; McIntosh, 2001; Studer et al., 2008), embedded, and sectioned for TEM examination. This procedure has been shown to preserve ultrastructural information better than chemical fixation (Kiss et al., 1990; McDonald and Morphew, 1993), but may fail for certain systems (Schertel et al., 2013). HPF specimens can also be sectioned and imaged by cryo-electron microscopy of vitreous sections (CEMOVIS) (Muller et al., 2008). While CEMOVIS produces remarkable results (Al-Amoudi et al., 2007; Bouchet-Marquis and Hoenger, 2011; Lucic et al., 2005; Medalia et al., 2007; Nicastro et al., 2005), it is as much an art as it is science, and it is fair to say that the barrier to entry is high. Finally, both freeze substitution and CEMOVIS suffer from microtome artifacts, such as uniaxial compression, knife-marks, and crevasses (Al-Amoudi et al., 2005; Bouchet-Marquis and Hoenger, 2011; Studer et al., 2008).

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Less demanding sample preparation, higher throughput, and the continuously improving resolution make field-emission scanning electron microscopy (SEM) a popular alternative to TEM methods. In a cryo-SEM, i.e. an SEM equipped with a cryostage and a loadlock, frozen-hydrated samples can be investigated without any chemical fixation, staining, or embedding. In order to reveal cellular ultrastructure and eliminate contamination, cryo-SEM is typically performed on freeze-fractured samples. Optionally, surfaces can be freeze-etched to enhance contrast between insoluble ultrastructural elements and the surrounding fluid phase, and coated with a metal (Heuser, 2011; Pawley, 2008). However, the propagation of the fracture plane cannot be controlled; the plane often propagates between the leaflets of lipid bilayer membranes and fails to reveal the interior of organelles (Branton, 1966). To ensure that structural information is obtained from a single plane only, and thereby facilitate morphometric studies, frozen-hydrated samples can be cryo-planed by cryo-ultramicrotomy (Huang et al., 1994; Walther, 2003; Walther and Muller, 1999). Even though many systems can be investigated using these two methods, mineralized tissues and other samples with high hard-soft contrast can be very challenging to prepare. This is because the fracture plane frequently responds in complex ways to the hard-soft interface, and hard and brittle minerals can damage the block face or even the knife during microtomy (Edwards et al., 2009; Volkert et al., 2004).

A much more surgical approach to investigate the ultrastructure of specific aspects of a sample is by focused ion beam (FIB) methods in a SEM (FIB-SEM) (Heymann et al., 2006, 2009; Schertel et al., 2013). In FIB-SEM and its cryo-variant, a highly focused beam of Ga^+ ions is used to mill away relatively small volumes; the surface so revealed can then be imaged, typically using back scattered electron contrast. FIB milling is not very sensitive to differences in material hardness and has been successfully used to cross-section composite systems (Friedmann et al., 2011; Wierzbicki et al., 2013). While this approach is very powerful and has been used to determine 3D ultrastructure by sequential 'slice-and-view' techniques (Bushby et al., 2011), cryo FIB-SEM equipment is very rare still, and quite costly. Moreover, sectioning by FIB is a time-consuming process that renders creating large cross-sections impractical. There would be an obvious advantage in combining the predictability and tolerance of hard/soft contrast of ion milling with the rapid preparation of large surfaces by freeze fracture. We reasoned that this should be possible by using broad ion beam milling, which additionally lowers costs significantly compared to FIB-based instruments. This principle was first explored for geological samples by Desbois et al., who chose an in situ approach where milling and freeze-etching are performed in a dedicated SEM (Desbois et al., 2012, 2013).

In this work, we describe the development of a stand-alone cryo triple ion gun milling (*CryoTIGM™*, pronounced cryo-"time") tool that creates large cross sections in high-pressure-frozen biological samples. As a proof of concept, we applied it to three systems, Baker's yeast, mouse liver biopsies, and sea urchin embryos. For comparison, we prepared in parallel the same samples by freeze fracture (FF). Analysis of cryo-SEM images taken from surfaces cryo-planed by *CryoTIGM™* revealed that *CryoTIGM™* compares favorably to freeze fracture and cryo FIB-SEM.

2. Materials and methods

2.1. Consumables

Dry Baker's yeast (*Saccharomyces cerevisiae*), lecithin (Whole Foods, Evanston, IL); sucrose (EMD Chemicals, Gibbstown, NJ); 1-Hexadecene (Sigma Aldrich, St. Louis, MO); Dulbecco's phosphate buffered saline (PBS) (Mediatech, Manassas, VA); platinum-coated double edge blades (Electron Microscopy Sciences, Hatfield, PA); 50-mL conical tubes, 60-mm petri dishes (VWR, Batavia, IL); grade 2 filter paper (Whatman-GE Healthcare Bio-Sciences, Pittsburgh, PA); liquid nitrogen, ultra-high purity argon gas (Airgas, Chicago, IL).

2.2. Preparation of tissues and cells

Dry Baker's yeast (150 mg) was suspended in a 5% (w/v) aqueous solution of sucrose (10 mL). Rehydration and activation, as judged by the evolution of CO_2 from the suspension, typically occurred within 20 min. Activated yeast was collected by centrifugation (6000 rpm; 4320*g*; 5 min), the supernatant removed, and the pellet gently stirred into slurry with a pipette tip.

Adult purple sea urchins (*Strongylocentrotus purpuratus*) were purchased from Bodega Marine Laboratory (Bodega Bay, CA). In vitro fertilization of the animals and the subsequent embryonic culture were performed as described before (Wu et al., 2011). At either the mesenchyme blastula/early gastrula stage (28–32 h post fertilization) or the pluteus stage (68–72 h post fertilization) of development, the embryos were harvested via centrifugation (15 °C, 750 rpm; 18*g*; 3 min). After removal of the supernatant, the pellet was gently pipetted up and down a few times to resuspend the embryos.

Mouse liver tissues were gifted by Dr. Thomas O'Halloran at Northwestern University. Tissues were harvested from female CD-1 mice (2–4 months of age) after they had been euthanized in the context of a different study, following approved protocols. Animals had been treated with pregnant mare serum gonadotropin and human chorionic gonadotropin, which do not affect normal liver physiology or function. Harvested liver tissue was placed in ice-cold PBS in a 50-mL conical tube and kept on ice. Using a surgical blade, tissue slices approximately 150 μm in thickness were prepared from liver lobes covered by ice-cold PBS. Discs (d = 1.9 mm) of the liver tissue were fashioned from these slices using a biopsy punch (#16770111133, Leica Microsystems, Vienna).

2.3. High-pressure freezing

For freeze fracture of suspended cells and embryos, the inside of aluminum specimen carriers (d = 3 mm, t = 100 μm or 200 μm, #16770141, Leica Microsystems, Vienna) was roughened with a needle to improve sample adhesion to the carrier. An aliquot (~0.6 μL) of freshly prepared sample was transferred into each of the two carriers (t = 100 μm). One carrier was then placed upside down on the other, effectively sandwiching the sample volume.

For freeze fracture of mouse liver, a tissue disc of less than 200 μm thickness was transferred into a carrier (t = 200 μm), and gently wicked with filter paper to remove the excess buffer. 1-hexadecene was then layered over the tissues. A second carrier (t = 100 μm) loaded with 1-hexadecene was placed upside down on the first one, creating the sample sandwich.

For *CryoTIGM™*, the sample sandwich was prepared as before, except that the first (bottom) carrier had a depth of 200 μm, and the sample volume was 1 μL. The second (top) carrier had a flat surface (#16770142, Leica Microsystems, Vienna) and was pre-coated with a 2% lecithin solution in chloroform for ease of removal after vitrification.

Assembled carrier sandwiches were immediately placed in a cartridge holder and cryo-fixed using a EM HPM100 high pressure freezer (Leica Microsystems, Vienna).