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Cryo-Planing of Frozen Hydrated Samples by Triple Ion Gun Milling (*CryoTIGM*)

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Cryo-fixation, i.e. the rapid vitrification of hydrated samples, omits the artifact-prone steps of chemical fixation, embedding, and dehydration, and consequently offers improved preservation of biological ultrastructure [1, 2]. Imaging of cryo-fixed samples by cryo-SEM is particularly efficient since time-consuming steps such as embedding and preparation of thin sections is not required. However, a fresh interior surface has to be prepared, typically by freeze fracture. The random nature of fracture does not ensure the passage of the fracture plane through the regions of interest [1]. Cryo-planing by cryo-FIB allows positional control of structural investigation, and smaller structures like single cells can be selected with surgical precision [3]. However, in many situations it would be advantageous to be able to cryo-plane larger areas of the frozen-hydrated samples. Herein, we describe a cryogenic sample preparation workflow for cryo-SEM based on broad ion-beam milling that addresses this issue.

The central innovation of the workflow of the cryo triple ion gun milling (*CryoTIGM*) method is a custom-built tool based on the Leica TIC3X slope cutter. Specifically, a TIC3X unit was fitted with a vacuum load lock that allows cryo-transfer of a vitrified sample. Samples were high pressure-frozen between aluminum planchettes and trimmed using a custom-built cryo-saw. The cryo-saw consists of a liquid nitrogen reservoir, a sample compartment, a diamond blade, and a VCT-docking port (Fig. 1A). Trimming was performed under liquid nitrogen, and samples were then positioned in a sample holder next to a milling mask (Fig. 1B). The sample was transferred to the *CryoTIGM* tool (Fig. 1C), where three broad Ar^+ beams converge at the mask shielding the trimmed sample edge (Fig. 1D). Material above the mask was removed, creating a cross-section in the sample at the level of the mask (Fig. 1E). The ion-milled sample was subsequently freeze-etched and coated with Pt to increase contrast.

We optimized operating parameters for *CryoTIGM* for a range of samples, including yeast suspensions, mouse liver biopsies, and suspensions of whole sea urchin embryos. Irrespective of the sample type, we find that ion milling with Ar^+ at an acceleration voltage of 3.0 kV, a current of 1.0 mA/gun, a base temperature of -120°C, and for 2 h results in very smooth cryo-planed area of ~700,000 μm^2 (Fig. 1E). Analysis of cryo-planed surfaces after freeze-etching and coating indicates that *CryoTIGM* does not induce crystallization of vitreous ice in well-frozen samples. A direct comparison of samples prepared by conventional freeze fracture and *CryoTIGM* revealed that 1) surfaces prepared by *CryoTIGM* are much smoother; 2) cellular and organellar details are observed at comparable high resolutions with good contrast; 3) most importantly, additional ultrastructural features can be revealed (Fig. 2). For example, in *CryoTIGM*-prepared sea urchin embryo samples, tight contacts among neighboring ectodermal cells, suggesting the presence of intercellular junctions, can be identified (Fig. 2B) Membrane tethers extended from ectodermal cells to the hyaline layer are visible. The hyaline layer is resolved to consist of two layers, with the space between them occupied by some kind of vesicles or granules.

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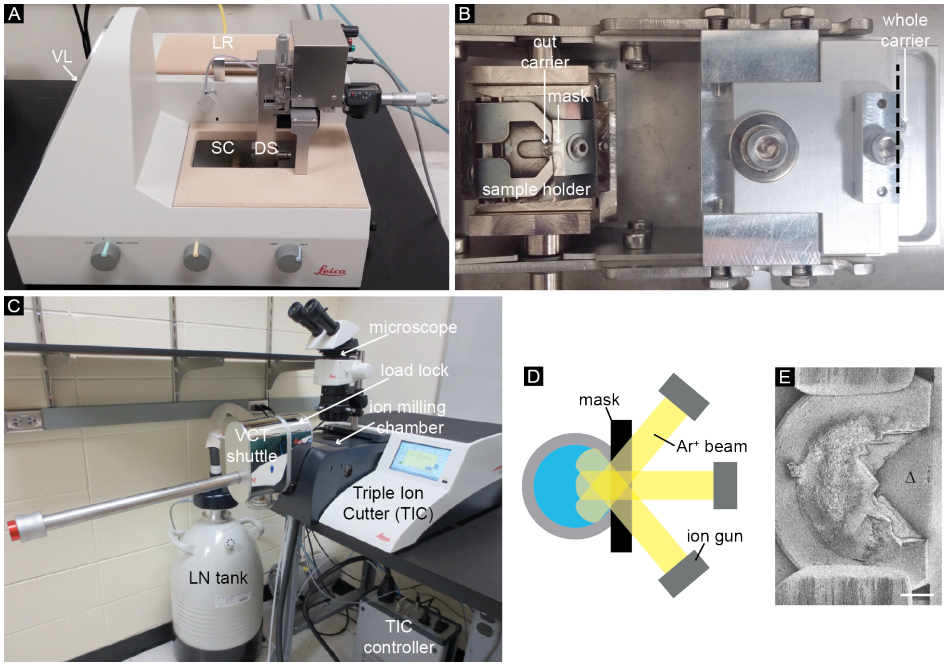

Figure 1. Experimental setup of *CryoTIGM*. (A) The cryo-saw used for trimming sample carriers. DS = diamond saw, LR = LN reservoir, SC = sample compartment, VL = VCT loading dock. (B) Top view of the sample compartment. A sample carrier is trimmed (along the dashed line) and transferred to the sample holder. (C) *CryoTIGM* tool with attached cryo/vacuum-transfer-shuttle. (D) Schematic drawing of *CryoTIGM* milling process. (E) A frozen hydrated sample after milling shows a triangular cryo-planed area of 700,000 μm^2 (Δ). Scale bar represents 500 µm.

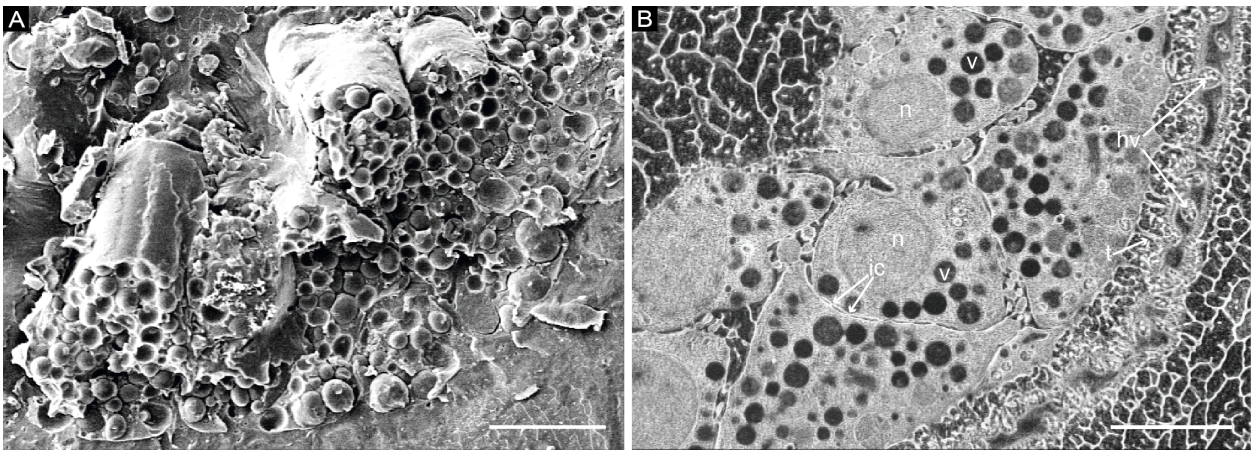

Figure 2. Cryo-SEM of high pressure frozen sea urchin embryos prepared by freeze fracture (A) and *CryoTIGM* (B). hv = hyaline layer vesicle, ic = intercellular contact, n = nucleus, t= tether, v = vesicle. Ice segregation is visible in the extracellular space and blastocoel. Segregation was observed in both freeze-fracture and *CryoTIGM* samples and is therefore an indication of poor vitrification, likely due to the high salt content (3.5 wt%) of the medium. Scale bar represents 5 µm.

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