doi:10.1017/S1431927622005177
Microsc. Microanal. 28 (Suppl 1), 2022
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# National Center for In-situ Tomographic Ultramicroscopy and the Waffle Method: An Approach for Cryo-FIB/SEM Thin Lamellae Preparation

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The National Center for In-situ Tomographic Ultramicroscopy (NCITU), along two other centers "spokes" and a central "hub", is part of an initiative to provide access and training to users interested in using Correlated Light Electron Microscopy (CLEM), cryo Focused Ion Beam – Scanning Electron Microscopy (cryo-FIB/SEM) and cryo-Electron Tomography (cryo-ET) as a method to answer biological questions. NCITU is located within the New York Structural Biology Center (NYSBC) as part of Simons Electron Microscopy Center (SEMC) and is funded under NIH Grant U24GM139171. As one of the three spokes, NCITU focuses on sample preparation and production of thin specimens suitable for cryo-ET. Tomographic data collection is performed by the central hub—Midwest Center for Cryo-Electron Tomography (MCCET). NCITU currently provides facilities for cell growth, target protein expression and live cell screening. Cells can be accurately positioned using a PRIMO micropatterning device and vitrified using Leica EM GP2 plunge freezer. Alternatively, cells can be applied at a high concentration to an EM grid and frozen using a Wohlwend high-pressure freezer (HPF) following the Waffle Method protocol.

The Waffle Method has been developed to address challenges within the conventional cryo-FIB/SEM workflow [1]. These challenges include low milling throughput, preferred orientation and poor vitrification of thick specimens. To address these issues, sample is applied on a grid which is then sandwiched between two planchettes and high-pressure frozen. After high-pressure freezing, the "waffle" grid is then clipped and inserted into a cryo-FIB/SEM instrument. A conductive platinum layer as well as a layer of GIS platinum is applied to the grid before milling. Lamella can then be prepared using a combination of manual and automated milling. Current applications include yeast, bacteria, and purified proteins. The manual milling preparation is done to clear away bulk material; this bulk material is first cleared using trenches that are milled orthogonally to the ion beam, separated by a distance of 25 µM. Then the grid is tilted in increments to clear away any bulk material underneath the site of the future lamella. A "notch" is then milled order to allow lamella stress relief. A template designed to automate milling waffle lamella is then used. Within a 8 hour day, two lamellae of approximately 160 nm and 12 µM width by 12 µM height are produced using this method.

References:

[1] K Kelley et al., bioRxiv 2020.10.28.359372 (2021).

https://doi.org/10.1017/S1431927622005177 Published online by Cambridge University Press