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On the Road to Correlative Cryo-Lift-Out, Fully Automated Waffles and Beyond – Make the Most out of your Tissue Sample

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Sample preparation for cryo-electron tomography is crucial in ensuring the best possible data quality to be mined for *in situ* structural biology [1]. Samples that are too thick prevent high-resolution analyses of individual molecular components using subtomogram averaging. In turn, samples that are too thin limit the volume that can be studied in such a way that the cellular context is no longer readily tangible.

The fabrication of cryo-FIB lamellae with gallium ions yields sufficiently large and homogeneously thin areas of biological samples from their original starting dimensions. The measure of machinability is the ability to sufficiently focus the gallium ion beam for artifact-free preparation, which in practice is limited to less than 50 micrometers of sample thickness [2]. This boundary affects not so much plunge frozen samples as it does to those frozen under high pressure (HPF) with an initial thickness of up to 200 microns. Waffles can facilitate access to HPF samples, but their initial ice thickness for direct milling of lamellae is still determined by the constraints imposed by gallium [3]. The extrication of lamellae currently seems to be the only way to access thicker samples such as tissues or whole organisms [4,5]. However, this lift-out procedure is not only very time-consuming, but also often disappointingly inefficient, primarily due to its complexity. Nevertheless, the success rate is crucial, especially for cellular and developmental biology aspects, since many tomograms are required to draw statistically relevant conclusions. This is equally true for macromolecular complexes that occur only sporadically.

It would be conceivable to make the lamellae larger to obtain more tomograms. However, the lateral size is limited by the mechanical properties of the vitreous ice, the milling geometry, along with the ablation rate and the physical properties of the ions used. For gallium, it therefore seems pointless to go beyond 30 microns, also in view of the low removal rates and the associated damage for prolonged milling times. Getting a grip on this would require novel hardware solutions, e.g. plasma sources with higher ablation rates and currents to make even 200-micrometer samples readily accessible. Moreover, one could even consider bypassing the error-prone step of cryo-lift-out (i.e., going ‘lift-less’) and get lamellae from HPF samples, in a similar and simple way as those produced from direct milling approaches.

In any case, the whole procedure for the fabrication of lamellae from HPF samples, whether with liquid metal ion source or plasma, needs to be streamlined and automated to become much more efficient. Here, we report on recent developments towards full automation of the Waffle milling procedure [6,7]. We also showcase the use of a correlative cryo-lift-out technique that combines an integrated fluorescence light microscope and a lift-out device in a single instrument [8,9]. Such a combined system cuts down a risky transfer and, most importantly, enables precise targeting of subcellular structures from

voluminous HPF samples. Ultimately, perspectives and preliminary results are presented on improving and eventually sidestepping cryo-lift-out, which would increase transfer efficiency, and in turn could make cryo-ET of multicellular organisms and tissues fairly routine.

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