

## Workflow for Correlative Cryo-Imaging

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The investigation of vitrified biological specimens enables the visualization of cellular ultrastructure in a near native fully hydrated state, unadulterated by harmful preparation methods. Therefore utilizing Light and Electron Microscopy in combination is the next step in the cryo-microscopy workflow to extract functional and structural information from the sample. Here, we focus on two recent cryo imaging modalities and discuss their impact on cryo-correlative workflows. First, we present confocal cryo fluorescence microscopy, utilizing a novel confocal detector scheme with improved signal to noise ratio (SNR) and resolution. Second, we show volume imaging of multicellular specimens by focused ion beam scanning electron microscopy (FIB/SEM) under cryo-conditions.

Confocal laser scanning microscopes (LSM) are renowned for their optical sectioning capability, a feature enabled by utilizing a pinhole that rejects out of focus light. Closing the pinhole improves lateral resolution, but also causes less light to reach the detector leading to reduced signal to noise ratios. In cryo-fluorescence microscopy, the situation is aggravated by the fact that currently no immersion optics are readily available and consequently only numerical apertures below NA 1 are possible. We combined Airyscan, a novel detector module (available for ZEISS LSM 780, 800 and 880) together with a cryo-correlative stage (Linkam CMS 196) for fluorescent imaging of vitrified samples. The Airyscan detection module allows the spatially resolved detection of fluorescence light otherwise rejected by the pinhole in a standard confocal system. We demonstrate that even without immersion optics, Airyscan achieves a significant increase in resolution and SNR compared to standard confocal images.

FIB/SEM tomography enables the acquisition large three-dimensional volumetric data from biological specimens by sequentially removing material with the ion beam and imaging the exposed block faces with the electron beam. This imaging method can be applied to frozen hydrated specimens, as recently demonstrated in (*J Struct Biol.* 2013 Nov; 184(2):355-60. doi: 10.1016/j.jsb.2013.09.024). In contrast to Cryo TEM Tomography (cryo-ET), FIB/SEM tomography allows easy mapping of large multicellular specimens in the near native state and is particularly suited to analyse samples that require vitrification. The trade-off compared to cryo-ET is a somewhat lower resolution comparable with resin-embedded samples. FIB/SEM volume imaging however elegantly extends cellular cryo-ET by providing much larger volume access at significantly reduced preparative labour and can thus add necessary contextual information that broadens the view possible with cryo-ET.

Both methods by themselves promise significant advantages for biomedical research by enabling the investigation of biological specimens in the near native fully hydrated state. Yet correlating both imaging modalities, LSM and FIB/SEM of vitrified samples, has the potential to provide even deeper insights into biological context. Our new software solutions i.e. ZEN Connect and Atlas 5 allow the user to perform one concise workflow between a ZEISS LSM and FIB/SEM instrument by keeping the various imaging organized spatially and at scale in one comprehensive project.

The correlation between cryo-light and electron microscopy data will greatly benefit from the ever-increasing resolution in fluorescence imaging. Cryo-Airyscan is the next step into that direction delivering three-dimensional optical sectioning data that enables reliable targeting of cellular structures in a FIB/SEM microscope. Once identified, the structural context of the target location either is explored by volume imaging or can be processed further as a cryo-lamella for subsequent cryo-TEM Tomography.