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Correlative Cryo Confocal Light Microscopy (C^3LM) and X-ray Fluorescence

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Correlative microscopy has been growing in importance as a way to obtain more complete information about a specimen than any one type of microscopy can provide [1]. For example, CLEM (correlative light and electron microscopy), which combines the strengths of both fluorescence light microscopy and electron microscopy, enables analysis of rare cellular (or subcellular) events in their cellular context. Fluorescence microscopy allows specific localization of rare moleculars or events in a sample, while electron microscopy reveals subcellular structure where they reside at nanometer scale spatial resolution. CLEM is traditionally performed in chemically fixed and dehydrated specimens. However, by using cryogenic sample preparation and imaging, frozen hydrated specimens with their subcellular structures preserved near native state have been used and become increasingly important [2,3,4,5]. Similar combinations of visible fluorescence and x-ray transmission imaging have been demonstrated on larger samples, owing to x-rays more favorable penetrative power [6]. We present here the development of a system with unique characteristics: the ability to mount a frozen hydrated specimen once, on a robust sample-handling cartridge, and image it under cryogenic conditions using both confocal light microscopy and x-ray fluorescence microscopy.

The handling of thin, delicate biological specimens under liquid nitrogen is not straightforward. That is why we have chosen to use the same specimen cartridge as is used in the Bionanoprobe (BNP) [7], a scanning x-ray fluorescence and transmission microscope operated at the Advanced Photon Source (APS) at Argonne National Lab (ANL). This microscope uses cartridges that can accommodate specimens mounted on 3 mm electron microscope grids and on 5 mm silicon nitride windows. Because the cartridge is manipulated by grabbing a base, it is in principle compatible with specimens prepared in thin-walled capillaries for 360° rotation angle tomography. Specimen mounting is done under liquid nitrogen (LN) or LN-cooled nitrogen gas, after which the cartridge is loaded into an evacuated transfer chamber with conductive cooling. Up to four cartridges can be transferred at once into the BNP, with an in-vacuum, cryogenically-cooled robotic transfer system used to load one cartridge at a time onto the microscope scanning stage. With the BNP, one can obtain sub-20 nm resolution x-ray transmission images using ptychography, and sub-100 nm x-ray fluorescence images of trace element distribution [8].

Our cryogenic confocal correlative light microscope (C^3LM) design concept is shown in Fig. 1. It uses the same cartridges and transfer chamber as the BNP from Zeiss, and makes significant use of sample transfer robot components purchased from Zeiss. With it, one can place a specimen cartridge on a conductively-cooled sample mount in the C^3LM vacuum system. The specimen can then be brought into the focal plane of one of several microscope objectives that can be rapidly exchanged without breaking vacuum, including a 0.8 NA, 100x dry objective (Nikon CFI TU Plan BD ELWD, with 4.5 mm working distance). In unpublished tests we carried out with Xradia it was demonstrated that one could obtain images with no discernable degradation in image quality when a cryogenic specimen was imaged with

this objective in vacuum, and the rest of the microscope optics out of vacuum on the other side of a vacuum viewport. Therefore the C^{3}LM system uses multiple standard objective lenses in vacuum, with a Nikon C2 confocal light microscope head located out of vacuum. The specimen stage is motorized for easy location of a feature of interest, and so as to allow for accurate indexing of tiled images to obtain a large field of view. The system is also being designed with push-button vacuum operation, and it will be located in a cryogenic sample preparation laboratory at ANL in close proximity to the BNP.

While it can be highly advantageous to integrate correlative microscopy capabilities into a x-ray microscope [6], there are several competing advantages to a seperate light microscopy instrument that is able to work with cryogenic specimens using the same mounting and exchange system. A separate light microscope can be accessed at any convenient time, independent of synchrotron beamtime scheduling. This allows one to evaluate specimen preparation protocols long before scheduled x-ray beamtime, and to identify the most interesting specimen regions far in advance. This in turn can make for more efficient use of x-ray beamtime. The use of confocal fluorescence microscopy also enables thicker biological specimens, and thus makes better use of one of the key advantages of x-ray microscopy over electron microscopy, namely the ability to image specimens tens of micrometers thick [9].

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Figure. 1. Mockup of the C^{3}LM. Center image shows the robotic transfer system [unpublished].

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