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Strategies for CLEM Imaging.

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Correlative Light and Electron Microscopy (CLEM) is a technique with many variations. The premise of the technique is to link events observable by fluorescence in cell biology and physiology with the higher resolution morphology and structure obtained by cryo TEM[1]. Thus once static snapshots from TEM now have a dynamic range. Our lab has developed strategies for correlating various biological systems for the CLEM imaging workflow[2]. We have developed a range of protocols for imaging labeled vs. unlabeled whole cells, locating labeled and expressed proteins within cells, transfected nanoprobes within cells, labeled phage attaching to bacteria, and virus attaching to and being taken up by cells. Each of these scenarios requires an adaptation to the sample preparation. In each case, the experiment needs to be designed such that it is compatible with plunge-freezing of the sample. A factor to consider is how the cells of interest will be adhered to the TEM grid. Some eukaryotic cells grow fine on carbon support films, while others require pre-coating of the film with substrates such as collagen or fibrinogen. Another factor is timing of application of transfection agent and potential washing steps. We then vitrify the cells on grids with either a Cryoplunge3 (Gatan) or a Vitrobot Mark III (FEI). Choice of blotting method also depends on the type and thickness of the cells of interest. Cryo fluorescence imaging (cryo-fLM) is done on a Leica cryo CLEM system (Leica Microsystems) with a 50 X ceramic objective (N/A=0.90)[3,4]. Areas of interest are mapped as multi-channel z-stacks using LAS X matrix screener software (Leica Microsystems). Coordinates of regions of interest within this grid map can be saved and then the map and coordinates are transferred to the JEOL JEM-2200FS 200 kV field emission TEM (JEOL, Ltd., Japan) equipped with an in-column Omega energy filter. Using the SerialEM software package [5] the fluorescence map and coordinates are imported and registered to the cryoTEM low magnification grid map of the same grid. From here the area of interest can by imaged at an intermediate magnification with SerialEM's montage option for morphological information, followed by higher magnification imaging or tomography.

One case study presented here is the use of a double-label system to detect HIV-1 particles internalized by CV-1/TVA950 cells. Upon fusion mCherry (red content marker) is instantly released from virions, thus only internalized virions are labeled green. CV-1 cells are grown directly on gold NH2 FinderTEM grids with Quantifoil R2/1 support film (Quantifoil Micro Tools GmbH, Jena, Germany) reinforced with the evaporation of a 4-5 nm layer of carbon using a Denton Benchtop Turbo apparatus (Denton Vacuum, Moorestown, NJ). The TEM grids are then placed into individual MatTek dishes with DMEM media and incubated overnight. CV-1 cells are then seeded directly onto the grids. Following overnight incubation, labeled virus is added and then washed off. Just prior to freezing, 10 nm colloidal gold in PBS is added to each grid for fiducial markers to aid in tomogram alignment. Grids are vitrified in liquid ethane using a Cryoplunge 3 System (Gatan, Pleasanton, CA) equipped with GentleBlot blotting pads.

Figure 1 illustrates the ability to localize a single internalized virion with cryo fLM. Fusion of the virion results in release of the mCherry label and visibility of the YFP-Vpr. Each virion is ~100nm and can be clearly located. Figure 2 illustrates the overlay of cryo fLM images with cryo TEM maps to precisely locate virions on and within the cell for further high resolution imaging.

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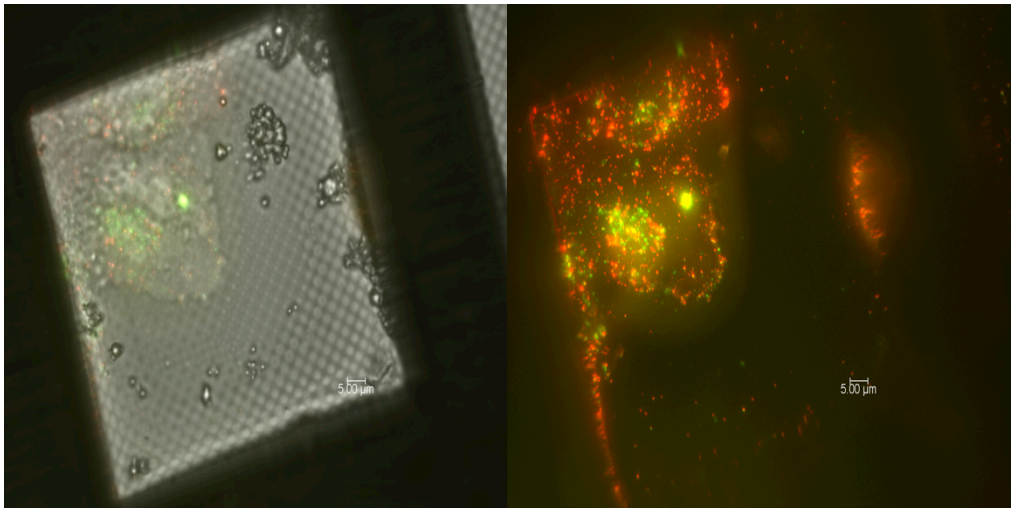

Figure 1. Cryo-fLM of double-labeled HIV-1 particles pseudotyped with Avian Sarcoma and Leukosis Virus (ASLVpp) Env glycoprotein. Fusion is manifested in instantaneous release of mCherry (red content marker) from virions. Scale bar 5um.

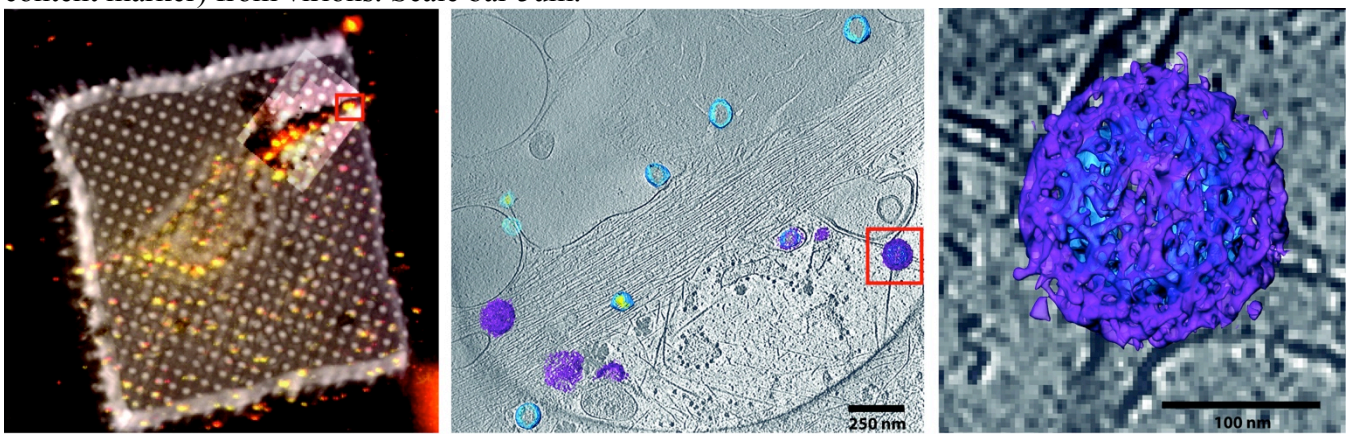

Figure 2. Cryo CLEM imaging of retroviral endocytosis and fusion. A) Cryo fLM of ASLV Env pseudotyped HIV-1 particles bound to CV-1/TVA950 cells. Upper-right corner is the overlay of the cryo-EM montage onto the cryo fLM image. Red square indicates the tomography data in panels B and C. B) Tomographic slice, with segmentation, of ASLV Env pseudotyped HIV-1 particles undergoing endocytosis. The viral membrane (light blue) and mature core (yellow) are rendered. Clathrin cages (purple) surround several viral particles. C) Enlargement of one clathrin cage (purple) surrounding a viral particle (light blue). Segmentation using Amira software (FEI). Scale bars: B) 250 nm, C) 100 nm.

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