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Imaging endosomes and autophagosomes in whole mammalian cells using correlative cryo-fluorescence and cryo-soft X-ray microscopy (cryo-CLXM)☆

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ABSTRACT

Cryo-soft X-ray tomography (cryo-SXT) is a powerful imaging technique that can extract ultrastructural information from whole, unstained mammalian cells as close to the living state as possible. Subcellular organelles including the nucleus, the Golgi apparatus and mitochondria have been identified by morphology alone, due to the similarity in contrast to transmission electron micrographs. In this study, we used cryo-SXT to image endosomes and autophagosomes, organelles that are particularly susceptible to chemical fixation artefacts during sample preparation for electron microscopy. We used two approaches to identify these compartments. For early and recycling endosomes, which are accessible to externally-loaded markers, we used an anti-transferrin receptor antibody conjugated to 10 nm gold particles. For autophagosomes, which are not accessible to externally-applied markers, we developed a correlative cryo-fluorescence and cryo-SXT workflow (cryo-CLXM) to localise GFP-LC3 and RFP-Atg9. We used a stand-alone cryo-fluorescence stage in the home laboratory to localise the cloned fluorophores, followed by cryo-soft X-ray tomography at the synchrotron to analyse cellular ultrastructure. We mapped the 3D ultrastructure of the endocytic and autophagic structures, and discovered clusters of omegasomes arising from 'hotspots' on the ER. Thus, immunogold markers and cryo-CLXM can be used to analyse cellular processes that are inaccessible using other imaging modalities.

1. Introduction

Correlative Light and Electron Microscopy (CLEM) is a powerful tool for identifying the subcellular localisation of fluorescent proteins within the reference space of the cell [1]. CLEM has been successfully applied to many studies of membrane trafficking in mammalian cells, including those involving the endocytic [2,3], recycling [4,5], secretory [6–8] and autophagy pathways [9–11]. However, the multi-stage sample preparation process has drawbacks. Chemical fixatives penetrate the sample too slowly to instantaneously preserve dynamic structures, heavy metal stains mask the atomic composition of the cell and dehydration of the specimen can cause deformation of the membranes. In addition, many consecutive (serial) sections must be cut, collected, imaged and reconstructed for 3D analysis of complex organelles.

Cryo-preservation techniques including plunge freezing (for samples less than 10 μm thick) and high pressure freezing (HPF; for samples less than 200 μm thick) are fast and circumvent the need for chemical fixatives, giving optimal sample preservation. Frozen samples may then be freeze-substituted into resin and observed at ambient temperature in a TEM (transmission electron microscope), or imaged directly at cryo-temperatures using

Abbreviations: CEMOVIS, Cryo-Electron Microscopy of Vitreous Sections; CLEM, Correlative Light and Electron Microscopy; Cryo-CLXM, Cryo-Correlative Light and X-ray Microscopy; Cryo-SXT, cryo-Soft X-ray Tomography; Cryo-TEM, cryo-transmission electron microscopy; ER, endoplasmic reticulum; FIB, Focused Ion Beam; GFP, Green Fluorescent Protein; HPF, high pressure freezing; PNR, perinuclear region; RFP, Red Fluorescent Protein; TfnR, transferrin receptor; VTC, vesicular–tubular clusters

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cryo-TEM, thus avoiding shrinkage artifacts caused by dehydration. However, sample thickness is limited to around 1 μm in a 300 kV TEM due to the penetration of the electron beam. Whereas studies of thin peripheral regions of intact cells are possible [12], studies of the thicker perinuclear region (PNR) in intact cells are not. Cryo-Electron Microscopy of Vitreous Sections (CEMOVIS) [13] or Focused Ion Beam (FIB) milling [14] can be used to view sections cut through the thicker perinuclear region (PNR) of frozen cells, which contains many of the cell's organelles, but imaging through an entire cell by either technique would be near-impossible.

Synchrotron-hosted cryo-soft X-ray tomography (cryo-SXT) is an emerging technique that allows imaging of unstained, cryo-preserved biological samples as close to their native state as possible [15–17]. Cells may be loaded into capillaries [18] or grown on carbon-coated grids [19,20]. Samples are then prepared by plunge-freezing, or more recently by HPF [21], both of which are highly effective at preserving dynamic and volatile cellular structures. Samples are then imaged in a cryo-SXT microscope at one of three synchrotron beamlines currently operational worldwide (the Advanced Light Source in the USA, BESSY II in Germany, ALBA in Spain). Image contrast is due to differential, quantitative absorption of X-ray photons in the 'water window', between the K-shell absorption edges of carbon (284 eV) and oxygen (543 eV) [19]. Here, organic materials show strong absorption and phase contrast, whereas water is relatively non-absorbing [22,23]. Fortuitously, at this energy, the X-rays penetrate through ~10 μm of ice. Therefore, whole mammalian cells can be imaged at near-native state, without chemical fixation, staining or sectioning.

Cryo-SXT has been applied to a range of sample types including yeast cells [24–28], mammalian cells [16,17,29–31] and virus-infected cells [20,32,33]. Ultrastructural preservation is similar to cryo-TEM of thin regions of frozen-hydrated cells [12] with the additional advantage of being able to image through the thickest part of the cell. Contrast is similar to TEM images of heavy-metal stained and resin-embedded cells, enabling identification of organelles including the nucleus, mitochondria, the Golgi apparatus and endoplasmic reticulum (ER) by their characteristic morphology [31]. However, organelles that may require immunogold labelling or CLEM for unambiguous identification in TEM, including endosomes and autophagosomes, have not yet been characterised in cryo-SXT images.

Recently, correlative studies combining cryo-fluorescence microscopy with cryo-SXT have started to link function to native-state ultrastructure, using either a cryogenic light microscope prior to cryo-SXT imaging [34–36], or a fluorescence microscope inside the X-ray microscope itself [19,20]. Here we report the use of a stand-alone cryo-fluorescence stage [37], which can be mounted on any standard widefield epifluorescence light microscope, for identification of fluorophore-labelled organelles using correlative cryo-fluorescence and cryo-soft X-ray microscopy (cryo-CLXM). We used cryo-CLXM to characterise structures of the endocytic and early autophagy pathway. Early autophagosomes can be difficult to localise, even using CLEM, as they are particularly susceptible to chemical fixation artifacts [38,39] and so would specifically benefit from a cryo-preparation and cryo-imaging approach.

To remain healthy, eukaryotic cells require a constant turnover and replacement of old components with functional new ones. Disposing of old organelles is a challenging task that cells tackle with autophagy, a lysosome-mediated degradative pathway. A key event in autophagy is the formation of a double-membrane structure called an autophagosome, which engulfs portions of cytosol and entire organelles. Autophagosome formation can be divided into five steps (with structure): induction (the pre-autophagosomal structure), expansion (the phagophore and the omegasome), vesicle completion (the autophagosome), fusion with the endo-lysosomal system (the amphisome) and cargo degradation by lysosomal enzymes (the autolysosome/lysosome) [40]. Autophagy specific proteins, called "Atg" proteins in yeast, can be used as markers for different stages of autophagosome formation. Atg9 is the only known transmembrane protein involved and is essential for the formation of early autophagosomal structures [41] whereas LC3 (Atg8) is cytosolic and is recruited to forming autophagosomes. Immunogold labelling [42] and CLEM revealed that Atg9 localises to complex vesicular–tubular clusters surrounding electron-lucent endosome-like organelles (Atg9-VTC) that interact with transferrin-receptor-positive endosomes [40]. However, despite fluorescence data indicating that Atg9-VTC co-locate with LC3-positive membranes, recognisable early autophagosomal structures were difficult to find and so our understanding of the early autophagy pathway remains incomplete.

In order to characterise forming autophagosomes in the vicinity of Atg9-VTC and endosomes, we developed a workflow for cryo-CLXM and also loaded the cells with an endosome-specific gold marker. We imaged co-localised RFP-Atg9 and GFP-LC3 signals in vitrified Hek293 cells using benchtop cryo-fluorescence microscopy, which also allowed us to screen samples for areas of suitable ice thickness. Using the HZB cryo full-field transmission X-ray microscope installed at the U41 beamline at the BESSY II electron storage ring (Berlin, Germany), we collected tomograms from the regions of interest in whole, unstained Hek293 cells. We identified early and recycling endosomes using an anti-transferrin receptor antibody conjugated to 10 nm gold particles (anti-TfnR-gold). Using our correlative workflow, we identified LC3-positive membranes that we postulate are omegasomes [43] due to their cup-shaped morphology. The omegasomes co-locate with TfnR-positive endosomes and Atg9-VTC, and for the first time we show multiple omegasomes arising in clusters from the same ER subdomain. Thus, cryo-CLXM and immunogold labelling can be used to identify the morphology of subcellular compartments close to their native-state, and reveal structure–function information about subcellular processes that are difficult to capture using traditional imaging techniques.

2. Materials and methods

2.1. Cells and constructs

The HEK293A/GFP-LC3/mRFP-Atg9 cells were previously described [10]. Cells were maintained in full medium (FM): DMEM with 10% foetal calf serum (FCS) as previously described [44,45]. To induce autophagy, cells were incubated for 2 h in starvation medium (Earle's balanced salt solution, EBSS). For identification of endocytic compartments, anti-TfnR-gold conjugate (10-nm gold) was added to the EBSS.

2.2. Holey carbon film preparation

HZB-2 gold grids (Gilder Grids, UK) were coated with holey-carbon films as a support for cell growth. Grids coated with 2/2 Quantifoil® film (Quantifoil, Germany) allowed uniform blotting prior to plunge freezing, and the holes were also found to be particularly useful for alignment during data reconstruction and correlation between imaging modalities. However, due to the expense and time taken to produce these grids, homemade films were also used. To make these, 20 drops of a 1:1 glycerol:dd H_20 solution were added to 50 ml of 0.5% (w/v) formvar in chloroform. The mixture was shaken vigorously for 30 s to disperse the aqueous glycerol into the solvent, and then sonicated using a microprobe sonicator for 60 s at 50% power, to reduce the glycerol microdroplet size. The solution was immediately transferred to a