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# Tools for correlative cryo-fluorescence microscopy and cryo-electron tomography applied to whole mitochondria in human endothelial cells

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## Abstract

Cryo-electron tomography (cryo-ET) allows for the visualization of biological material in a close-to-native state, in three dimensions and with nanometer scale resolution. However, due to the low signal-to-noise ratio inherent to imaging of the radiation-sensitive frozen-hydrated samples, it appears oftentimes impossible to localize structures within heterogeneous samples. Because a major potential for cryo-ET is thereby left unused, we set out to combine cryo-ET with cryo-fluorescence microscopy (cryo-FM), in order to facilitate the search for structures of interest. We describe a cryo-FM setup and workflow for correlative cryo-fluorescence and cryo-electron microscopy (cryo-CLEM) that can be easily implemented. Cells are grown on finder grids, vitally labeled with one or two fluorescent dyes, and vitrified. After a structure is located by cryo-FM (with 0.4 μm resolution), its image coordinates are translated to cryo-ET stage coordinates via a home-built software routine. We tested our workflow on whole mount primary human umbilical vein endothelial cells. The correlative routine enabled us to investigate mitochondrial ultrastructure for the first time on intact human mitochondria, and led us to find mitochondrial cristae that were connected to the intermembrane space via large slits, which challenges the current view that such connections are established exclusively via small circular pores. Taken together, this study emphasizes that cryo-CLEM can be a routinely used technique that opens up exciting new possibilities for cryo-ET.
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***Keywords:*** Correlative microscopy; Cryo-fluorescence microscopy; Cryo-electron microscopy; Cryo-electron tomography; Mitochondria; Endothelial cells

## Introduction

Cryo-electron tomography (Cryo-ET) is rapidly expanding in the life sciences as a technique that allows for high-resolution 3D imaging of biological samples in a close-to-native state of preservation (Koster et al., 1997; Leis et al., 2009). Samples for cryo-ET are immobilized by rapid cooling down to cryogenic temperatures. The rapid cooling, which can be achieved for instance by high-pressure freezing or by plunge-freezing in liquid-nitrogen-cooled ethane, vitrifies the water in and around the sample. If a sample is thin enough (up to a few hundred nanometers), it can be viewed directly by cryo transmission electron microscopy (cryo-EM) and submitted to cryo-ET without any

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further processing (for a review, see Koning and Koster, 2009). Thus, the technique has been applied to several thin mammalian cell types (Briegel et al., 2008; Cyrklaff et al., 2007; Koning et al., 2008; Kurner et al., 2004). If samples are thicker, additional processing is required, such as cryo-ultramicrotomy (Al-Amoudi et al., 2004) or focused ion beam milling (Marko et al., 2007). Contrast in cryo-EM and cryo-ET is provided by local differences in molecular density of the sample. As a consequence, the differential contrast is much lower than in chemically fixed samples where contrast is improved by post-fixation and staining with electron-dense heavy atoms (e.g. osmium, uranium, lead). The low differential contrast is worsened by the low electron-dose requirements inherent to the technique. For the localization of structures of interest in cryo-EM, the lack of contrast is not a problem when the sample under investigation consists of a homogeneous population of structures, so that any area in the sample will be equally suitable for cryo-ET. It can become problematic, however, when a sample is heterogeneous in one or more aspects. To give but three examples, (*i*) a sample can consist of cells that were genetically modified but only a subpopulation expresses the modification, (*ii*) the features of interest can be infrequently occurring or short-lived events and therefore difficult to capture and find, or (*iii*) a sample can contain thicker and thinner parts, only the latter being thin enough for cryo-ET. In scenarios like these, it is not uncommon that one can determine only after tomographic reconstruction whether an appropriate area of interest was recorded. It would therefore be extremely helpful if cryo-ET could be preceded by an imaging technique that does not cause radiation damage but allows for the pinpointing of areas of interest for subsequent cryo-ET. A technique that could achieve this is correlative light and electron microscopy (CLEM).

CLEM refers to a series of techniques that aim at correlating light microscopic images with electron microscopic images acquired from exactly the same location in a sample (Valentijn et al., in press). Although CLEM studies have been published for over three decades, recent advances in fluorescence microscopy techniques, live-cell imaging, and fluorescent probe development have rekindled interest in CLEM research. The primary purpose of CLEM is to combine the advantages of light microscopy (generates overviews, large palette of stains and labels available, time-lapse recording) with those of electron microscopy (imaging at nanoscale resolution and in 3D, Koster and Klumperman, 2003). Thus it becomes possible to study the dynamics of a biological process and subsequently identify the ultrastructural determinants underlying that process (van Rijnsoever et al., 2008; Verkade, 2008). In addition, CLEM can greatly facilitate the identification of areas of interest for ultrastructural analysis in complex samples (Agronskaia et al., 2008; van Driel et al., 2008; Vicidomini et al., 2008). To have this option available for cryo-ET, it is necessary that the light microscopy be performed at cryogenic temperatures. Because standard light microscopes are not designed for working at cryogenic temperatures, special equipment is required. Furthermore, an accompanying cryo-CLEM workflow is needed to keep the sample vitrified at all times during the procedure, and to establish a multimodal coordinate system for fast retrieval of areas of interest.

Recently, two separate cryo-CLEM approaches have been described in the literature, each including a home-made cryo-FM setup (Sartori et al., 2007; Schwartz et al., 2007). Here, we report our approach to cryo-CLEM, which was developed in parallel. Our cryo-FM setup is centered around a commercially available heating and freezing stage that can be mounted on any conventional upright light microscope. Only two modifications made it suitable for use in cryo-CLEM: (*i*) the specimen holder of the stage was adapted for carrying EM grids with vitreous samples, (*ii*) a specimen loading box filled with liquid nitrogen vapor was attached to the stage to minimize frost buildup during grid transfers. Finder grids were used to provide reference points, and a coordinate translation routine was developed in Matlab to retrieve in cryo-EM structures of interest that were selected in cryo-fluorescence mode. The resolution of the cryo-fluorescence optics (0.4 μm) as well as the positioning accuracy of the translation routine (0.5 μm) are superior in comparison with the earlier reports. Thus, we believe that our approach excels in ease of implementation, convenience in use, and resolution of the cryo-fluorescence setup.

Using our cryo-CLEM approach, we examined, for the first time in primary human cells, the 3D ultrastructure of intact (i.e. non-sectioned) and fluorescently labeled mitochondria. Our results challenge the current view on mitochondrial membrane architecture: whereas the lumina of cristae are thought to connect to the inter-membrane space only via small circular openings, we show that the openings can also be elongated and slit-like.

## Materials and methods

### Sample preparation

Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, USA) were cultured as described previously (Valentijn, K.M. et al., 2008). Briefly, cells were grown in Endothelial Growth Medium-2 (EGM-2; Lonza) on gold EM finder grids (Agar Scientific, Essex, England). The grids were of type HF15 (135 mesh) or type H6 (100 mesh) and were coated with Formvar and

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