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High-precision correlative fluorescence and electron cryo microscopy using two independent alignment markers ☆

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ABSTRACT

Correlative light and electron microscopy (CLEM) is an emerging technique which combines functional information provided by fluorescence microscopy (FM) with the high-resolution structural information of electron microscopy (EM). So far, correlative cryo microscopy of frozen-hydrated samples has not reached better than micrometre range accuracy. Here, a method is presented that enables the correlation between fluorescently tagged proteins and electron cryo tomography (cryoET) data with nanometre range precision. Specifically, thin areas of vitrified whole cells are examined by correlative fluorescence cryo microscopy (cryoFM) and cryoET. Novel aspects of the presented cryoCLEM workflow not only include the implementation of two independent electron dense fluorescent markers to improve the precision of the alignment, but also the ability of obtaining an estimate of the correlation accuracy for each individual object of interest. The correlative workflow from plunge-freezing to cryoET is detailed step-by-step for the example of locating fluorescence-labelled adenovirus particles trafficking inside a cell.

1. Introduction

Electron cryo microscopy (cryoEM) is a powerful technique to visualise molecules in their native state. The method consists of imaging biological samples in the frozen-hydrated state at cryogenic temperatures [1–3]. Vitrification, i.e. rapid freezing of specimens within an amorphous, non-crystalline, glass-like ice layer, allows the preservation of structures down to the molecular level in the native, hydrated state. This is of special importance for cellular samples that are sensitive to chemical fixation and dehydration [4,5]. CryoEM has been used to study a vast range of specimens, from isolated macromolecules to the complexity of a cell. Despite these tremendous capabilities, particular for cellular cryoEM, one major limitation remains: locating the subcellular event of interest with nanometre-scale precision on the three-millimetre-diameter, frozen-hydrated, EM grid.

A very promising approach to achieve this goal is to use position information from fluorescence microscopy. This method employs, for example, fluorescently labelled proteins, to assist with their subsequent location and identification in EM. This methodology is typically referred to as correlative light and electron microscopy (CLEM). Initially introduced for specimens at ambient or cultivation temperature (for recent reviews, see [6,7]), more recently it has been applied for frozen-hydrated specimens [8–10]. CLEM relies on a two-step approach. First, the regions of interest (ROI) are imaged and located with light and fluorescence microscopy techniques. Secondly, the samples are imaged in EM, and the ROIs are assigned based on the fluorescence data. Most proteins can be tagged with a fluorophore, and analysed specifically with a wide range of light microscopy techniques such as live-cell imaging, confocal microscopy, super-resolution, e.g., stimulated emission depletion microscopy (STED) [11], or localisation microscopy [12–14]. The samples are either imaged by fluorescence microscopy before fixation (correlation with live-cell imaging), or after fixation. Expectedly, only post-

Abbreviations: Ad5-488, Adenovirus serotype 5 labelled with Alexa-488; CLEM, correlative light and electron microscopy; cryoEM, electron cryo microscopy; cryoET, electron cryo tomography; cryoFM, fluorescence cryo microscopy; EM, electron microscopy; FM, fluorescence microscopy; FWHM, full-width at half-maximum; HMM, high magnification montages; LM, light microscopy; LMM, low magnification montages, LN_2, liquid nitrogen; LWD, long working distance; NA, numerical aperture; PSF, point spread function; ROI, region of interest; STED, stimulated emission depletion microscopy; σ^A, accuracy of alignment; σ^C, accuracy of fluorescent fiducial based coordinates transformation; σ^E, accuracy of locating event of interest; σ^T, total accuracy of correlation

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fixation imaging allows for the confident correlation of fast, dynamic processes with EM, such as cell mobility, cytoskeleton dynamics, virus infection, and endocytosis. This limitation may be reduced by the use of rapid cryo immobilisation preparation procedures such as high-pressure freezing [15]. Most CLEM procedures use a two-step imaging workflow with a transfer step between microscopes dedicated for a particular modality (light microscopy (LM) or EM), as presented in this study. However, dedicated combined instruments which avoid the need for sample transfer exist [16,17]. CLEM was initially introduced for the analysis of sections of plastic embedded material, such as resin embedded samples or Tokuyashu type cryo sections in methyl cellulose. The technique was more recently applied to frozen-hydrated specimens, such as plunge-frozen purified complexes, and for guiding cryoET on whole vitrified cells [9,16,18–21].

Electron microscopists have made considerable efforts to facilitate the correlation across several orders of magnitude. For instance, special EM grids marked with numbers or letters recognisable in low magnification greatly assist the first steps of correlation. In most studies performed on plastic embedded samples, the fluorescence information only guides the correlation at a rather low-resolution scale, such as certain parts of a cell, and the re-location of the ROIs in EM remains somewhat approximate. Invasive immunolabelling or post-fixation contrasting methods are often used in order to better assign position information for the labelled molecules [22,23]. Only recently, a method using fluorescent electron-dense microspheres as CLEM fiducials achieved a correlation in the range of 100 nm, solely guided by fluorescence [24,25]. In these studies the bleed through from the FluoSpheres signal into the channel of the fluorescent protein of interest had been used to correct for shifts between the channels. With the approach used, the authors were able to follow the location of endocytic vesicles in resin embedded sections of yeast cells and to describe the endocytic process across several stages [26]. This impressive demonstration of the use of fluorescent fiducials for resin embedded samples poses the question of its applicability for cryo specimens.

Indeed, cryoLM has the great advantage to provide the location of the feature of interest at the exact same position on the EM grid without the need to compensate for sample deformation caused by the shrinkage and warping of plastic sections due to electron beam irradiation. At the same time, cryoLM/FM represents a greater challenge than conventional LM/FM imaging for a number of reasons. Firstly, the sample needs to be imaged whilst being kept below the de-vitrification point at about −135 °C [27]. With the development of dedicated LM cryo sample stages it became possible to perform such experiments [8,10,18–20]. Secondly, with the exception of in-column fluorescence microscopy implementations [16], the sample needs to be cryo-transferred to the electron microscope for subsequent EM imaging. This poses two risks, (i) the risk of damaging or losing the specimen, and (ii) the risk of ice contamination. Thus, in comparison to the substantial body of literature reporting the application of correlative studies on resin embedded samples, only a few studies have reported correlative cryoLM/EM. Moreover, correlation accuracy significantly below the micrometre precision has yet to be achieved [28].

Here, we describe a solution to improve the precision of correlative cryoLM/EM of plunge-frozen specimens. We present a workflow implementing (i) TetraSpeck fluorescent microspheres as markers for multi-channel cryo fluorescent image correction (as proposed in [24]), and (ii) fluorescent electron dense microspheres as reference points for an accurate correlation and transfer of coordinates between LM and EM. Besides giving an overview of the workflow, a detailed description of the practical steps is provided. The power of this approach is illustrated with a biological example; the high-precision location of adenovirus particles within infected cells.

2. Materials and methods

2.1. Purification and labelling of virus particles

First generation E1/E3-deleted Adenovirus serotype 5 viral vectors (Ad5) were produced by infecting twenty 15 cm dishes of HEK 293 cells (ATCC CRL-1573™) at a ratio of 100 physical particles per cell. Cells were continued to grow for 36 h until detachment was apparent. Cells were collected, pelleted and virus was extracted by freeze-thaw cycles and purified using double CsCl2-banding. Purified Ad5 particles were labelled using the Alexa-488 microscale protein labeling kit (Invitrogen) using the manufacturers protocol as described in detail in Martinez et al. [29] (Ad5-488).

2.2. EM grid preparation, infection of cells grown on EM grids

Gold finder EM grids with regular patterned holey carbon films (Protochips CF-2/1-2F1-Au) were glow-discharged for 20 s and coated by submersion in 100% poly-L-lysine solution (Sigma-Aldrich) for 30 to 60 min. Subsequently, the grids were coated with fluorescent microspheres that were used as fine correlation markers, *i.e* carboxylate-modified blue fluorescent FluoSpheres (350Ex/440Em) of 0.1 μm diameter, (Invitrogen). The application of a 1/4000 diluted FluoSphere solution directly onto the carbon support of the EM grid followed after 5 min with a washing step with PBS yielded the required density of ~5–15 FluoSpheres per high-magnification EM image. Subsequently, human bone osteosarcoma epithelial cells (U2OS) were grown on the grids in Dulbecco's modified medium (Invitrogen), supplemented with 10% (w/v) fetal calf serum (Sigma-Aldrich) for 15 to 24 h before infection. The growth medium was replaced after 10 to 15 h to remove unattached cells and cell debris. Fluorescently labelled adenovirus particles were adsorbed to human osteosarcoma cells (U2OS) at 37 °C for various amounts of time. Prior to vitrification 1 μl of 15 nm colloidal gold particles (Aurion) and 2 μl of a 1/20 diluted solution of TetraSpeck microspheres (0.2 μm diameter, showing four well-separated excitation/emission peaks, 360/430 nm (blue), 505/515 nm (green), 560/580 nm (orange) and 660/680 nm (dark red), Invitrogen) were added directly onto the grid. The density of TetraSpeck microspheres must be low enough to allow the detection of the green fluorescence of adenovirus particles (Ad5-488) in the FM, but high enough that sufficient fluorescent markers (~5–15) are found in a single grid square. Excess of buffer was removed by manual blotting from the non-carbon side of the grid, and cells were vitrified by plunge-freezing in a liquid nitrogen-cooled ethane/propane mixture [30]. Samples were stored submersed in liquid nitrogen for later analysis.

2.3. Fluorescence cryo microscopy

For fluorescent microscopy, an AxioObserver Z1 inverted microscope (Zeiss) equipped with 20× (NA 0.4) and 63× (NA 0.75, working distance 1.7 mm) air objectives, a motorised stage (Märzhäuser), and a monochrome AxioCam MRm camera (Zeiss) was used. A mercury arc lamp (HXP) was used for fluorescent excitation with the following band pass filter cubes (AHF): 482/18 (Ex), 520/28 (Em) (green); 560/40 (Ex) and 630/75 (Em) (red); 387/11 (Ex), 447/60 (Em) (blue). Zeiss AxioVision 4.3 software was used to control the microscope stage, filter cubes, shutters and camera. Fluorescent and phase contrast imaging at cryogenic temperatures was performed as described previously [19]. Briefly, a cryostage^2 cryo sample stage (MPI Biochemistry, Martinsried [18]) was mounted onto the light microscope's motorised stage. This setup employs a liquid nitrogen (LN_2) automated pumping system (Norhof LN_2 microdosing system series 900). Samples were transferred from liquid nitrogen storage only when the temperature of the microscope cryo sample stage was