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Coordinate transformation based cryo-correlative methods for electron tomography and focused ion beam milling

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ABSTRACT

Correlative microscopy allows imaging of the same feature over multiple length scales, combining light microscopy with high resolution information provided by electron microscopy. We demonstrate two procedures for coordinate transformation based correlative microscopy of vitrified biological samples applicable to different imaging modes. The first procedure aims at navigating cryo-electron tomography to cellular regions identified by fluorescent labels. The second procedure, allowing navigation of focused ion beam milling to fluorescently labeled molecules, is based on the introduction of an intermediate scanning electron microscopy imaging step to overcome the large difference between cryo-light microscopy and focused ion beam imaging modes. These methods make it possible to image fluorescently labeled macromolecular complexes in their natural environments by cryo-electron tomography, while minimizing exposure to the electron beam during the search for features of interest.

1. Introduction

Vitrification of biological material provides excellent sample preservation that is suitable for imaging by electron microscopy (EM). Methods such as plunge-freezing and high-pressure freezing cause rapid cooling and vitrification of the sample and preserve its hydration, while lipid membrane disruption, ionic concentration changes and protein structure alterations are prevented [1,2]. Consequently, transmission electron microscopy (TEM) imaging of fully hydrated, vitrified samples can provide high-resolution information about isolated molecules and complexes (cryo-EM), or three-dimensional (3D) images of macromolecular complexes and other cellular components with a resolution of 2–4 nm, even when imaged within their cellular environments (cryo-ET) [3,4]. Because structure-based identification of molecular complexes imaged within their cellular environment is possible only for larger complexes at the present resolution level, other approaches, including correlative methods, are required to help with molecular identification and cryo-EM navigation to features of interest [5]. The navigation to features of interest is also needed when vitrified samples of 5–10 μm in thickness, such as cell cultures, have to be thinned by focused ion beam (FIB) milling [6–8] in order to make them thin enough (in the range of 0.5 μm) for the subsequent imaging by cryo-ET.

The widespread use of fluorescence microscopy and the large repertoire of currently available fluorescent probes has provided insights into the function and spatial distribution of a large number of proteins and has greatly advanced our understanding of cellular processes. Fluorescence microscopy complements cryo-ET because it allows the unambiguous identification of fluorescently labeled proteins. However, only a few labeled species can be imaged simultaneously, yielding images in which the context of labeled proteins remains invisible. The combination of light microscopy (LM) with cryo-ET facilitates the search to features of interest, which otherwise can involve extensive scanning, and may allow correlation of fluorescently labeled proteins with features visualized in cryo-tomograms.

LM imaging of vitrified samples (cryo-LM) assures that features of interest are identical during LM and EM imaging, thus avoiding the problem of possible alterations arising prior to and during vitrification. This imaging mode was made possible by the development of specialized cryo-holders mountable on standard light microscopes [9–11]. In contrast to this modular approach, a closer integration of light and electron imaging offers more streamlined handling [12] or the use of higher numerical aperture objectives [13], but requires specialized instrumentation, and is less flexible than the modular approach.

Early applications of correlative LM and cryo-ET took advantage of strong visual cues provided by large cellular features, which allowed a straightforward orientation of the sample [14,15]. The lack of distinct morphological features in complex, densely populated cell cultures requires a purely computational, coordinate

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transformation-based approach. A coordinate transformation is established that is subsequently used to correlate coordinates of features of interest at LM images to TEM stage coordinates or coordinates at cryo-EM images [11,16].

Here, we continue the development of the coordinate transformation based methods that correlate features of interest identified by LM of vitrified samples to two other imaging modes. First, we describe a more efficient method that can be used to navigate the cryo-EM search to the feature of interest, which does not rely on imaging special features of EM grids such as corners between grid bars, because these can be obscured by cells grown on the grid or by a thick layer of vitrified material. Second, we extend our method in order to correlate cryo-LM features of interest to FIB images that define the location where thin vitrified lamellae are produced by FIB milling [17]. In both cases, an improved cryo-LM stage [7] was used for LM imaging of vitrified samples both in phase-contrast and fluorescence modes.

2. Materials and methods

2.1. Cell culture, labeling, and plunge-freezing method

Gold Quantifoil grids (R1/4, NH2 finder grid, Quantifoil Micro Tools, Jena, Germany) were used as a substrate for BHK-21 cell cultivation. Before use, each grid was cleaned by floating on acetone. Then, an additional carbon coating ($\approx$15 nm) was applied on the film side of each grid by a carbon evaporator (MED 020, BAL-TEC). Next, grids were sterilized by UV irradiation for 30 min and immersed in 1 mg/ml poly-L-lysine/0.1 M borate buffer (pH 8.5) in the dark overnight. After poly-L-lysine coating, grids were washed with autoclaved distilled water, and kept in Ca^{2+} and Mg^{2+} free HBSS-HEPES at room temperature until used for cell culture. BHK-21 (baby hamster kidney) fibroblast cells were cultivated in minimum essential medium (MEM) containing Earle's salts and L-glutamine supplemented with 10% FBS, non-essential amino acids (NEAA), and 100 U/ml penicillin and 100 μg/ml streptomycin (all chemicals are from Invitrogen). BHK-21 cells were cultured in a CO_2 incubator at 37 °C and 7% CO_2. About 1.0×10^5 cells were seeded on EM grids placed one in each well of four-well 35 mm diameter cell culture dishes.

For mitochondrion labeling, BHK-21 cells were labeled with Mitotracker FM green (Invitrogen) by incubating with 200 nM staining solution for 45 min in a CO_2 incubator. Cells were then washed with pre-warmed culture medium two times. For Golgi apparatus labeling, BHK-21 cells were incubated with 30 particles per cell (PPC) of CellLight® Golgi-GFP, BacMam 2.0 (Invitrogen) for 16 h in the CO_2 incubator.

For comparative live and cryo-fluorescence, HeLa cells (passage number 15–20) were plated on poly-D-lysine coated grids at the density of 1.5×10^5 cells per plate (2 ml of media per plate). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS. Twenty-four hours after plating, cells were transfected with EGFP (3 μg DNA/plate) using Lipofectamine 2000 (Invitrogen; 9 μl Lipofectamine/plate). DNA and Lipofectamine 2000 were both diluted in Opti-MEM. Transfection was performed according to manufacturer's instructions. Four hours after adding the DNA/Lipofectamine 2000 mixture, normal growth media was added back to the plates. Cells were harvested for 48 h after transfection.

Four μl of pre-warmed fluorescent beads (Invitrogen F8887, 1 μm diameter) diluted 1:100 in HBSS-HEPES were applied to some grids. EM grids were vitrified by plunge-freezing in a liquid ethane/propane mixture at close-to-liquid nitrogen temperature using a Vitrobot® (FEI, Eindhoven, The Netherlands). The blotting chamber conditions were set to 37 °C, 90% humidity, and 10 s blot time. After plunging, excess ethane/propane mixture was blotted from the grid placed in the liquid nitrogen surrounding the ethane cup, by using a small piece of filter paper. Grids were stored in liquid nitrogen until usage.

2.2. Cryo-LM imaging

Plunge-frozen grids were mounted in grid support rings (Autogrid™ sample holder, FEI), either in the standard type for TEM observations, or into the support rings modified for FIB milling [17]. Grids were then loaded on the improved version of cryo-LM holder, which offers lower contamination (and thus longer imaging times) and is suitable for shorter working-distance LM objectives than the original design [7]. For cryo-LM imaging after FIB-milling and tomography grids mounted in grid support rings were removed from TEM cartridge holders and transferred to the cryo-LM holder. All steps were done under cryogenic conditions.

Cryo-LM imaging was performed on an Axiovert 200M (Zeiss, Oberkochen, Germany) epifluorescence microscope, equipped with a motorized stage (DC 120 × 100, Märzhäuser, Wetzlar, Germany), CCD camera (AxioCam MRm, Zeiss), a short-wavelength mercury lamp, and fluorescent filter sets (e.g. filter set 38 HE, Zeiss and filter set F46-005, AHF analysentechnik AG). Z-stack images of grids were acquired using the "MosaiX" module of the Axiovision software (Zeiss, Version 4.9.0) with LD Achroplan 40 × /0.60 Korr Ph2 objective lens (Zeiss).

Images used for comparative live and cryo-fluorescence were acquired using identical settings for all images (20 × objective lens magnification, 500 ms exposure time, no digital gain). Z stacks were acquired at increments below 5 μm and best focused positions were used for analysis. The integrated fluorescence intensities were measured in ImageJ and were background subtracted.

2.3. Cryo-EM and ET

Grids mounted in grid support rings were transferred into the TEM cartridge holder for Polara as described earlier [17]. TEM imaging of vitrified samples was performed with a FEI Polara microscope operated at 300 kV. The microscope was equipped with a field emission gun, a 2k × 2k Gatan CCD camera, a Gatan post-column energy filter operated in the zero-loss mode and a computerized cryo-stage designed to maintain the specimen temperature below −150 °C. Tilt series were collected using FEI tomographic acquisition software from −60° to 60° with 2° angular increments. Pixel size was 0.71 nm at the specimen level and the defocus was set to −8 μm.

Tilt-series were reconstructed by the weighted back-projection method, using the free software package IMOD version 4.5.7 (http://bio3d.colorado.edu/imod/) [18]. Final alignment of the tilt-series images was performed using the linear interpolation option in IMOD, with a low pass filter (cut off, 0.35; sigma, 0.05). No CTF correction was performed on either dataset. Aligned images were binned to the final pixel size of 2.852 nm. For tomographic reconstruction, the radial filter options were left at their default values (cutoff, 0.35; fall off, 0.05).

Accuracy of the TEM stage movements was determined by a series of stage movements starting from and returning to the same initial position, so that the initial and final stage coordinates were the same. Relative displacement of the images recorded at the initial and the final positions represented the stage error.

2.4. FIB and scanning electron microscopy (SEM)

SEM and FIB imaging and FIB milling were carried out on a dual-beam (FIB-SEM) microscope (Quanta 3D FEG, FEI) equipped with a custom-built 360° rotatable cryo-stage operated below −180 °C. Grids mounted on autogrid rings were placed on cryo-FIB shuttle [7]