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Correlated cryo-fluorescence and cryo-electron microscopy with high spatial precision and improved sensitivity

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ABSTRACT

Performing fluorescence microscopy and electron microscopy on the same sample allows fluorescent signals to be used to identify and locate features of interest for subsequent imaging by electron microscopy. To carry out such correlative microscopy on vitrified samples appropriate for structural cryo-electron microscopy it is necessary to perform fluorescence microscopy at liquid-nitrogen temperatures. Here we describe an adaptation of a cryo-light microscopy stage to permit use of high-numerical aperture objectives. This allows high-sensitivity and high-resolution fluorescence microscopy of vitrified samples. We describe and apply a correlative cryo-fluorescence and cryo-electron microscopy workflow together with a fiducial bead-based image correlation procedure. This procedure allows us to locate fluorescent bacteriophages in cryo-electron microscopy images with an accuracy on the order of 50 nm, based on their fluorescent signal. It will allow the user to precisely and unambiguously identify and locate objects and events for subsequent high-resolution structural study, based on fluorescent signals.

1. Introduction

Fluorescence microscopy (FM) can provide information on the position and dynamics of specific, fluorescently labeled molecules during biological processes. Multiple molecules can be distinguished from one another by the use of fluorophores of different colours. Molecules can be chemically labeled, or fluorescent proteins such as green fluorescent protein (GFP) [1] can be genetically encoded and expressed as part of the protein of interest. This approach has generated tremendous insights into cell biology [2]. Due to fundamental optical limitations, the spatial resolution of conventional FM is limited to a few hundred nanometers. Technological approaches to overcome the diffraction limit ("super-resolution" methods) allow the organization of labeled proteins to be studied with a resolution reaching tens of nanometers [3,4]. However, FM visualizes the fluorescent tag, and does not directly provide information on the structural and morphological context in which the fluorescent tag is observed.

Electron microscopy (EM) can be used to obtain a detailed view of the molecular architecture of a cell at ultrastructural resolution. This information can be extended to three dimensions by acquiring several images of the sample from different angles and computationally reconstructing the imaged volume in a method called electron tomography (ET). EM and ET are ideal techniques to analyze the structure of macromolecular complexes or the morphology of cellular compartments and structures. EM would benefit from the application of specific labels that could be used to identify particular proteins of interest. Approaches to specifically label molecules with antibodies [5], or electron-dense tags [6–8] are, however, challenging, and often interfere with the structural preservation of the sample.

Correlative light and electron microscopy (CLEM) approaches aim to combine the advantages of FM and EM to locate specific events or transient intermediate states and to study the underlying ultrastructure. They thereby combine the power of FM to identify specifically labeled structures in a large area, and the power of EM to reveal the morphology and molecular architecture of the region of interest.

Many different approaches have been taken to correlate information from FM and EM. In one set of approaches, FM is carried out before the sample is prepared for EM. For example, live-cell fluorescence imaging has been used to identify transient intracellular features of interest [9–11] or cells of a specific genotype. Fluorescence signals have been used to target sites within multicellular organisms for subsequent EM [12,13]. In these approaches

Abbreviations: CLEM, correlated light and electron microscopy; EM, electron microscopy; FM, fluorescence microscopy; GFP, green fluorescent protein; LN, liquid nitrogen; NA, numerical aperture; WD, working distance

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there is typically a time gap of at least a few seconds between the states observed in the FM and EM images, corresponding to the time taken to fix or immobilize the sample after fluorescence imaging. In this time the biological process may proceed, and the object of interest may move. Distortions during sample preparation introduce further differences between the sample seen in the FM and that seen in the EM [14]. For these reasons, combining live-cell imaging with later preparation of the EM sample is not ideal for the study of small features and fast moving processes. In another set of protocols, the sample is prepared for EM prior to carrying out FM imaging. In this case, there are no biological changes in the sample between the two imaging modalities, only distortions induced during transfer between microscopes or upon exposure to the electron beam. CLEM approaches in which both EM and FM are carried out on resin-embedded samples [15–17], must find a compromise in the sample preparation procedure between optimally preserving sample structure, and optimally preserving the fluorescent signal. The right compromise depends on the biological specimen, but in all cases the use of embedding resins and heavy metal stains prevents achieving molecular structural resolution. Alternatively, the sample can be prepared by vitrification, and FM can be carried out on the vitrified sample: this is cryo-FM. Maintaining the sample in a close-to-native, vitrified, hydrated state gives optimal structure preservation, and the potential to obtain molecular detail by cryo-EM.

To perform cryo-correlative microscopy (cryo-FM/EM), there exist several prerequisites. The most crucial one is, that at all times after vitrification, the sample temperature has to remain below −140 °C to avoid formation of crystalline ice. The fluorescence microscope therefore has to be equipped with a special cryo-stage that maintains low sample temperatures during fluorescence imaging. Similarly important is that the cryo-stage minimizes contamination of the sample due to frosting from environmental humidity. To avoid the detrimental effects of vibrations or drift on the fluorescence image quality, the stage must have sufficient mechanical and thermal stability. The stage should also allow imaging on standard 3.05 mm diameter EM grids or cartridge systems that can be transferred to cryo-electron microscopes. An optimal cryo-stage and associated workflow would furthermore allow high-resolution imaging, achieve high-sensitivity in order to image low numbers of fluorophores, and would allow high-accuracy correlation (better than 100 nm). This goal can only be reached using high-performance optics, most notably an objective with the maximum numerical aperture (NA).

A number of cryo-stages and associated workflows have been developed and successfully applied to carry out correlative cryo-FM/EM. The reader is referred to Briegel et al. [18] for a detailed review of this subject. The setup developed by Sartori et al. [19] uses an inverted microscope, meaning that the objective is positioned to observe the sample from below. With this instrument the authors were able to image labeled filament bundles in migrating keratinocytes that they could then locate and characterize in cryo-EM. This setup makes use of a microscope objective with a long working-distance (WD), separated from the sample by a glass slide. An improved version of this system employed a 0.75 NA objective with a 2 mm WD [20]. This development allowed fluorescent labels in yeast cells to be localized. A major advantage of the system is that the samples remain in the identical cartridge throughout all imaging steps including cryo-EM.

A second system, developed by Schwartz et al., makes use of an upright microscope in which the objective approaches the sample from above [21]. The imaging chamber is cooled using nitrogen gas evaporating from a dewar of liquid nitrogen (LN). Using this setup it was possible to identify microtubule bundles at the periphery of mamallian cells by cryo-FM and analyze their structure and organisation by subsequent cryo-electron tomography. Photobleaching of the fluorophores was found to be significantly reduced under cryo conditions. This system also requires use of long- WD objectives, with an NA of up to 0.7 providing an estimated resolution of 0.6 μm. An improved version of this system, with the capability of loading multiple grids at once, was also designed [18].

A third system, also designed for upright microscopes, was developed by van Driel et al., adapting a commercial stage produced by Linkam [22]. The stage is cooled by LN that is sucked by a pump through a cooling line that passes through a silver block supporting the sample. This circulation leads to evaporation of the LN within the block, providing efficient cooling. The original design of the stage features a cover with a glass slide separating the sample from the objective. However, this can be modified to allow the objective to enter the chamber and face the sample directly. This modification permits use of an objective with an NA of 0.75, allowing visualisation of, for instance, stained mitochondria in plunge-frozen endothelial cells. An associated transfer box aids in loading vitrified samples.

A contrasting strategy is to insert an optical system into the electron microscope, and to carry out both FM and EM in a single instrument. To switch from EM to FM mode, the grid is tilted by 90 degrees into the optical axis and the FM microscope is inserted into the EM column [23]. This approach eliminates sample transfers and thus minimizes the risk of contamination or devitrification [24]. The spatial limitations within the EM column limit the size of FM objective that can be used, and the system operates with a 0.55 NA objective.

All of the discussed setups have in common that they make use of dry lenses with relatively long WDs. For geometrical reasons, such objectives have a limited NA. As the amount of collecting photons is increased with the squared inverse sine of the NA, the NA is the most crucial factor determining the sensitivity of fluorescence observations. Furthermore, it is also critical to the spatial resolution. Objectives with higher NA can be used together with a liquid immersion medium at very low temperatures [25], but this is technically challenging and not broadly used. An alternative is to minimize the WD to maximize the NA of the objective and thereby the optical resolution and sensitivity that can be achieved.

So far we have discussed approaches for collecting FM and EM images of the same sample. Once the images have been collected it is necessary to relate them to one another. The position of a fluorescent object of interest can be localized within an FM image with high precision by applying a Gaussian fit to the fluorescent spot. Next, the position of the FM signal of interest must be identified within the EM image. This can be achieved using image correlation, landmarks on the grids, or fiducial markers within the sample. The most accurate positional prediction can be achieved by using fiducial markers: these are sub-diffraction limit sized objects that can be visualized in both FM and EM. Image transformations, calculated based on the coordinates of the fiducial markers in both images, can account for changes in scale and rotation. Notably, these transformations can also compensate for geometrical distortions that happen during moving the sample from one microscope to the other or due to the initial exposure to the electron beam [16,17]. Employing a high number of fiducial markers assures a reliable global coordinate transformation between the two images. Using the transform calculated from fiducial markers, the position of a spot of interest signal within the EM image can be identified with an accuracy of < 100 nm.

Here we introduce an adaptation of the cryo-stage described by van Driel et al. [22] that allows use of high NA objectives, and that is optimized to reduce ice contamination and improve ease of use. We then describe and demonstrate a workflow using fiducial markers to achieve very high-precision FM/EM correlation for vitrified samples.

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