Journal of Structural Biology 197 (2017) 83–93

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

New hardware and workflows for semi-automated correlative cryo-fluorescence and cryo-electron microscopy/tomography

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 17 June 2016
Accepted 27 June 2016
Available online 28 June 2016

Keywords:
Correlative light and electron microscopy
Cryo-fluorescence microscopy
Cryo-electron microscopy
Cryo-electron tomography

ABSTRACT

Correlative light and electron microscopy allows features of interest defined by fluorescence signals to be located in an electron micrograph of the same sample. Rare dynamic events or specific objects can be identified, targeted and imaged by electron microscopy or tomography. To combine it with structural studies using cryo-electron microscopy or tomography, fluorescence microscopy must be performed while maintaining the specimen vitrified at liquid-nitrogen temperatures and in a dry environment during imaging and transfer. Here we present instrumentation, software and an experimental workflow that improves the ease of use, throughput and performance of correlated cryo-fluorescence and cryo-electron microscopy. The new cryo-stage incorporates a specially modified high-numerical aperture objective lens and provides a stable and clean imaging environment. It is combined with a transfer shuttle for contamination-free loading of the specimen. Optimized microscope control software allows automated acquisition of the entire specimen area by cryo-fluorescence microscopy. The software also facilitates direct transfer of the fluorescence image and associated coordinates to the cryo-electron microscope for subsequent fluorescence-guided automated imaging. Here we describe these technological developments and present a detailed workflow, which we applied for automated cryo-electron microscopy and tomography of various specimens.

1. Introduction

Correlative light and electron microscopy (CLEM) combines the advantages of fluorescence microscopy (FM) and electron microscopy (EM). FM provides positional as well as dynamic information on specific biomolecules. EM provides detailed, high-resolution information on cellular ultrastructure and protein structure, while also revealing the environment surrounding the molecule of interest. This makes EM and its extensions to 3D volume imaging a powerful tool for structural biology as well as the method of choice for detailed analysis of cellular morphology (Carroni and Saibil, 2016; Lučič et al., 2013).

A biological sample needs to undergo preparatory steps before it can be imaged by EM. The goal is to maintain the sample in a close-to-native state and ensure optimal structure preservation while enabling it to enter the high vacuum microscope column. In "traditional" EM the sample is dehydrated and embedded in resin prior to staining with heavy metals and imaging at room temperature. In contrast, in cryo-EM the sample is imaged in its fully hydrated, vitrified state. The samples can be prepared by quickly plunging them into ultra-cold liquefied gases (Dubochet et al., 1988; Taylor and Glaeser, 1974), or by freezing them under high-pressure (McDonald, 2009). For samples that are too thick for cryo-EM imaging (above ~500 nm), thin slabs can be cut from

Abbreviations: CLEM, correlative light and electron microscopy; CEMOVIS, cryo-electron microscopy of vitrified sections; EM, electron microscopy; ET, electron tomography; FM, fluorescence microscopy; FOV, field of view; LN, liquid nitrogen; NA, numerical aperture; POI, position(s) of interest; TEM, transmission electron microscopy; WD, working distance.

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http://dx.doi.org/10.1016/j.jsb.2016.06.020
1047-8477/

the sample by cryo-sectioning (Al-Amoudi et al., 2004) or the sample can be milled with a focussed ion beam (Rigort et al., 2012). Contrast is generated directly by the electron density differences within the molecules. Hence, cryo-EM permits the morphological and structural characterisation of biological samples near to their native state and at high-resolution (Carroni and Saibil, 2016). In electron tomography (ET), 2D projection images acquired at different specimen angles are combined to generate a 3D reconstruction of the imaged volume (Irobalieva et al., 2016; Lučič et al., 2013).

In recent years various CLEM techniques using traditional sample preparation methods have provided remarkable insights into different areas of cell biology (Briggs and Lakadamyali, 2012; Bykov et al., 2016; Gibson et al., 2014; Redemann and Müller-Reichert, 2013; Sjollema et al., 2012). To maximize the accuracy with which fluorescence signals are identified in an EM image, both FM and EM need to be performed on the identical specimen (Kukulski et al., 2012, 2011). This raises a particular challenge in correlating FM with cryo-EM: the FM imaging needs to happen at temperatures below −140 °C where the cryo-EM sample does not devitrify. In addition, the sample must be shielded from atmospheric humidity during imaging and loading, while mechanical motion and optical distortions due to cooling or temperature gradients must be minimized. A number of solutions have been developed that add a special cryo-stage to a standard or adapted fluorescence microscope for cryo-CLEM (Zhang, 2013). Currently available solutions that fit an inverted fluorescence microscope feature a glass slide that separates the cold sample from the microscope objective lens (Sartori et al., 2007; Schellenberger et al., 2014). The minimum working distance (WD) reported for an inverted cryo-stage system is 410 μm with a 0.9 numerical aperture (NA) objective lens (Arnold et al., 2016). Solutions that attach to an upright microscope geometry do not require a separating glass slide (Liu et al., 2015; van Driel et al., 2009). The optical performance of all of these systems is limited by use of an immersion-free objective lens with relatively high WD and correspondingly low NA (Briegel et al., 2010; Koning et al., 2014). In a previous study using an upright geometry, we have shown that a short-WD dry objective lens with a high NA can be used for high-accuracy cryo-CLEM with a specially designed stage in which the objective approaches the sample closely (Schorb and Briggs, 2014).

We have developed a new dedicated cryo-FM system for CLEM. Our main goals during development of this system were: 1) to improve the transfer of the sample to and from the cryo-stage to reduce contamination by atmospheric moisture and to avoid sample loss due to warming or otherwise failed transfers; 2) to provide a mechanically and thermally stable stage appropriate for imaging multiple samples over long periods of time; 3) to maximize the optical performance; and 4) to incorporate the new hardware and its control software into a workflow that allows automated FM scans of entire cryo-EM grids and easy transfer of FM images and coordinates to the EM for use during data collection. Here, we describe and assess the new cryo-FM system. We provide a detailed workflow and protocol for using the cryo-FM system to automatically image an entire EM grid; for transfer of coordinates from FM to the software controlling the electron microscope; for cryo-EM or cryo-ET data acquisition; and for the image registration steps required to maximize the accuracy of correlation.

2. Results

2.1. Design of the stage and transfer shuttle

The cryo-FM stage is designed such that it can be mounted on a commercial upright microscope body (DM6FS, Leica Microsystems, Wetzlar, Germany). In this configuration the stage is fixed in *Z* but can move in *X* and *Y* directions, while the objective is inserted into the stage from above and moves axially for focusing. An overview of the stage and transfer shuttle design is given in Fig. 1. Inside the stage, liquid nitrogen (LN), provided by an external pump, cools a metal block that supports the specimen. The interior of the stage is thermally isolated from the stage casing by the use of non-conductive materials. A high NA (0.9), short WD objective enters the stage through a port in the stage lid that tightly surrounds the objective (Fig. 1G). The objective and the enclosing lid move relative to the flat stage surface during lateral (*X* and *Y*) movements while maintaining a seal to prevent influx of air. The front part of the objective is made of ceramic material with little thermal conductance to minimize heat transfer to the sample during imaging (Fig. 1C). To avoid condensation inside the objective from environmental humidity, the spring mechanism for the front lens assembly was removed and the whole objective lens was sealed. In addition, a glass slide was added in the parfocal adapter that connects the objective lens and the microscope body to avoid build-up of condensation on the objective back lens. There is no intermediate cover glass between the objective and the sample, allowing the front lens to approach to 280 μm from the sample.

The sample is loaded into the stage using a transfer shuttle that can be mounted onto the side of the stage (Fig. 1A, B, E). The transfer shuttle is filled with LN that cools the loading assemblies and provides a cold, dry environment to transfer samples from storage containers to the cryo-FM stage and vice versa. A transparent lid with spacers covers the transfer station when not in active use.

Inside the transfer shuttle, the vitrified samples on cryo-EM grids are loaded into a specialized, commercial, flat, square, copper cartridge for cryo-FM imaging (Leica 16707511109) (Movie S1). The transfer shuttle contains three spaces for standard cryo-EM grid storage boxes (Fig. 1D). A grid can be removed from a storage box and mounted into the cryo-FM cartridge using a dedicated loading station. In the loading station thin copper clips that secure the grid inside the cartridge (Fig. 1D) are raised, allowing the grid to slide into its position within the cartridge, and lowered to secure it in place. These copper clips have minimized material thickness in order to allow the objective to approach the sample as closely as possible. (Other available cartridge systems for cryo-EM secure the grid using a clip ring assembly whose thickness prevents FM imaging at low WD.) This configuration provides mechanical stability and protection during loading and handling of the sample. The grid stays mounted in the cartridge during transfer and imaging. The cartridge loading station is detachable and can be baked-out separately from the transfer shuttle.

The transfer shuttle incorporates an extendable rod with a gripper at its tip into which the cartridge can be inserted. To transfer the sample into the cryo-FM, the shuttle is docked to the stage, the rod is extended through a port in the wall of the shuttle directly into the cryo-FM stage and the gripper is released, leaving the cartridge in the stage (Fig. 1F). The transfer shuttle is removed before imaging. During imaging the cartridge sits on top of an LN-cooled copper block. The design is such that vibrations from the pump or the evaporation of LN are minimized. A temperature sensor regulates the pumping rate and LN flow. A heater element is embedded in the copper block for adjusting the temperature and for baking out the system after use. A hole in the block underneath the specimen allows brightfield imaging. The vaporising dry nitrogen gas creates an overpressure atmosphere in the stage and prevents contamination by frost from condensing atmospheric humidity.

The microscope is controlled using a dedicated CLEM module that we integrated into Leica's existing MatrixScreener HCSA (High Content Screening Automatization) software. This module provides the required functionality for generating grid-wide scans of cryo-EM specimens. The generated data format allows a direct exchange