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Marker-free method for accurate alignment between correlated light, cryo-light, and electron cryo-microscopy data using sample support features

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ABSTRACT

Combining fluorescence microscopy with electron cryo-tomography allows, in principle, spatial localization of tagged macromolecular assemblies and structural features within the cellular environment. To allow precise localization and scale integration between the two disparate imaging modalities, accurate alignment procedures are needed. Here, we describe a marker-free method for aligning images from light or cryo-light fluorescence microscopy and from electron cryo-microscopy that takes advantage of sample support features, namely the holes in the carbon film. We find that the accuracy of this method, as judged by prediction errors of the hole center coordinates, is better than 100 nm.

1. Introduction

Correlative imaging of samples using fluorescence markers to identify macromolecular assemblies and structural features in images and volumes obtained by electron cryo-microscopy approaches has become an important tool for biomedical research in recent years. In electron cryo-microscopy (cryo-EM), samples are vitrified by fast freezing, resulting in the biological samples being embedded in a layer of amorphous ice, preserved in their native state (Dubochet et al., 1988). Recent hardware and software developments have revolutionized the field, allowing now to obtain structural information at near atomic resolution for a wide variety of macromolecular assemblies (Subramaniam et al., 2016). The same advances also enable imaging of unperturbed cellular landscapes at high resolution (Asano et al., 2015a). Although some large macromolecular assemblies are identifiable in such cellular reconstructions (Asano et al., 2015b; Mahamid et al., 2016; Woodward et al., 2015; Fukuda et al., 2017) the majority of the components are not identifiably without specific labeling, presenting a major challenge in crowded cellular environments.

The advantages of fluorescence microscopy are complementary to those of electron cryo-microscopy. The fluorescence microscopy approach allows live cell imaging and to follow specific fluorescently labeled molecules in real time. The larger field of view also facilitates the identification and localization of sparse cellular events in a larger context. The combination of the two microscopy approaches potentially allows identifying regions associated with specific biological events by fluorescence microscopy and then investigating the underlying structural features at high resolution by cryo-EM.

For correlative imaging, vitrification of the sample can be either performed before or after fluorescence imaging. The advantages of room temperature fluorescence imaging include the use of optimized equipment such as high-powered, oil-immersion lenses with large numerical apertures and that dynamic processes can be characterized and followed. Even if precautions are being made to avoid imaging induced damage related to buffer and anti-oxidative agents, the disadvantage of this procedure is that structures may change or that the biological process of interest may be completed in the time required to perform the vitrification. To avoid this problem, cells can be fixed with carefully chosen fixation protocols to minimize artifacts that might be caused by cross-linking of cellular components. For fluorescence imaging after vitrification, specialized cryo-stages that allow fluorescence imaging at cryo-temperatures are required (Sartori et al., 2007; Briegel et al., 2010; Faas et al., 2013; Hampton et al., 2017; Arnold et al., 2016) and the resolution is restricted to about 400 nm or less owing to lack of immersion cryo-objectives. In addition, cryo-fluorescence imaging loses the capability of capturing the dynamics of the biological process. Correlative imaging using super-resolution techniques and electron cryo-microscopy are also under development (Wolff et al., 2016; Nahmani et al., 2017).

For all these correlative techniques, it is necessary to find an accurate alignment between the images from the two imaging modalities. The general workflow for this type of correlative imaging involves acquisition of fluorescence images and acquisition of cryo-EM images at increasing magnifications towards a final high magnification image or tilt series for tomographic reconstruction. The coordinate transform for the first step, i.e. correlating the fluorescence image with a low-

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magnification cryo-EM image, generally involves in-plane rotation, translation, and scaling. In some instances, when there are significant beam-induced changes to the sample (Brilot et al., 2012) or the sample support is tilted significantly in respect to the second imaging modality, further correction may be necessary. The subsequent steps are less complex for several reasons: (i) There is no change in rotation between the subsequent cryo-EM images; (ii) the difference in magnification is well defined, (iii) because the imaging modality does not change, features correlate very well between the successive images because one is simply a lower-resolution version of the other. Highly accurate methods for alignment of cryo-EM images based on fiducial markers (Kremer et al., 1996) or correlation (Winkler and Taylor, 2006) are well established in the cryo-EM field.

Early applications of correlative fluorescence/cryo-EM imaging took advantage of large cellular features to correlate fluorescence images with low-magnification cryo-EM images, which allowed a straightforward relative orientation of the sample (van Driel et al., 2009). To increase the accuracy of the alignment and to allow determination of accuracy estimates, the use of fluorescence beads as fiducial markers for alignment was introduced (Kukulski et al., 2011; Schorb and Briggs, 2014; Schellenberger et al., 2014). Here, we propose a marker-free approach using features of the sample support to provide alignment between images from fluorescence or cryo-fluorescence microscopy and cryo-EM images. The alignment quality of this approach is comparable to that previously reported for marker-based alignment and does not involve modification of the experimental procedures.

2. Materials and methods

2.1. Sample preparation

GFP:paxillin-transfected mouse embryonic fibroblast and Chinese hamster ovary cells (Bachir et al., 2014) were cultured on electron microscopy grids as previously described (Anderson et al., 2016). *Streptococcus pneumoniae* samples were prepared as previously described (Durand et al., 2015). Grids were manually plunge frozen in liquid ethane at liquid nitrogen temperature either after room temperature fluorescence imaging and mild fixation and/or before cryo-fluorescence imaging.

2.2. Correlative fluorescence and electron cryo-microscopy

We tested the alignment protocols using samples on commonly used electron microscopy grids (Quantifoil Grids with R1.2/1.3, R1/4 or R5/20 hole patterns). Fluorescence modalities used for correlated imaging included FRET, confocal, and cryo-fluorescence imaging with mCherry or GFP, recorded with a variety of objectives and camera pixel sizes. Cryo-fluorescence imaging was performed using a CorrSight CLEM microscope (FEI Company), equipped with a motorized stage, a broadband Xenon light source and motorized fluorescent filter sets with details previously described (Arnold et al., 2016). The CorrSight contains a 40 X objective and the camera pixel size is 161 nm. The vitrified grids were mounted into grid support rings (Auto-gridTEM sample holder, FEI) and transferred into the cartridge holder of a CorrSight CLEM microscope for imaging. The clipped grids are then transferred into the Titan Krios electron microscope so no manual grid handling between cryo-fluorescence and cryo-EM imaging is necessary. Cryo-EM images were acquired with an FEI Titan Krios transmission electron cryo-microscope (FEI Company) equipped with an extra-high brightness field emission gun and operated at 300 kV using a back-thinned 4 k × 4 k FEI Falcon II direct detection device. Images used for hole identification were taken at magnifications between 500× (pixel size 28 nm) and 1500× (pixel size 9.3 nm) depending on the magnification of the correlated fluorescence images and the density of the hole-pattern on the grid. The defocus was individually adjusted to emphasize the edges of the holes as much as possible.

2.3. Identification of holes in the carbon film

To obtain estimates for the center positions of the holes in the images we applied the following multi-step procedure.

(i) An iterative median filter (van der Heide et al., 2007) was applied to suppress background noise while preserving the sharpness of the hole edges. We used 20 iterations, which is usually enough to reach the stationary fixed point (Tyan, 1981). This step is fully automatic.
(ii) The optimal threshold for binarization was determined interactively.
(iii) The Canny edge detector (Canny, 1986) was applied to the binarized image. This step is automatic.
(iv) An initial estimate of the hole-radius was obtained interactively either from the binarized image or from the edge image.
(v) The circular Hough transform (Kimme et al., 1975) was run on the edge image with variable radius around the initial radius to determine the optimal radius for detecting the holes. High values in the circular Hough transform image correspond to centers of circular objects in the transformed image.
(vi) The circular Hough transform was re-run with the optimized radius.
(vii) The optimal number of peaks was determined interactively using a simplified peak search. The determined number of peaks was extracted using a full-blown, high-quality peak search algorithm. These peaks correspond to the center coordinates of the detected circles.

2.4. Alignment using the circle center coordinates

To allow the use of the circle center coordinates for alignment, we first needed to determine the mapping of the same holes in the two different imaging domains. The first step is to pick a small number of correspondences interactively (a minimum of two). This is achieved by giving each peak a unique label, rotating the fluorescence image approximately in the same orientation as the cryo-EM image and then displaying the two labeled images next to each other and to the corresponding reference images. Picking a small number of correspondences is then straightforward. Once initial correspondences were determined, these were used to automatically find all other correspondences between the two images. Initial scales were automatically determined using all distances between all holes and their correspondences. Using this scale estimate, initial estimates of the in-plane rotation and the relative translation were obtained using simplex optimizations (Nelder and Mead, 1964) every 45° and picking the one that resulted in the lowest root-mean-square deviation (rmsd) between the reference center coordinates and the corresponding transformed coordinates in the other imaging domain.

As a second step, all parameters i.e. scale, in-plane rotation, and relative translation are refined by minimizing the rmsd using the simplex optimizer. The entire workflow has been implemented in the pyCoAn framework, a python-based extension of the CoAn package (Volkmann and Hanein, 1999; Volkmann, 2009). A complete run takes about 5–10 min on a modern workstation. The software is freely available and can be accessed, together with documentation and tutorials, at the pyCoAn website (coan.burnham.org).

2.5. Simulated data

A hole pattern was created in the GNU Image Manipulation Program (www.gimp.com). The rest of the operations were performed within the pyCoAn framework. A collection of large interspersed Gaussian spheres of different sizes and weights were created and projected to emulate a cell-like density. The resulting image was overlaid with the hole pattern. The image was scaled down by a factor of six, rotated by 255° and