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Correlated Cryo-fluorescence and Cryo-electron Microscopy with High Spatial Precision and Improved Sensitivity

Martin Schorb¹ and John A. G. Briggs^{1,2}

¹Structural and Computational Biology Unit, European Molecular Biology Laboratory, D-69117 Heidelberg, Germany

²Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, D-69117 Heidelberg, Germany

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.

Keywords

cryo-fluorescence microscopy, cryo-FM; correlative light and electron microscopy, CLEM; cryo-electron microscopy; high-accuracy localization; fiducial beads; bacteriophage particles; low-temperature fluorescence microscopy

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 multi-cellular organisms for subsequent EM [12,13]. In these approaches 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 and co-workers [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 and colleagues, 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 mammalian 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 and colleagues, 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 NA squared, 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.

2 Results

2.1 The cryo-stage

The setup used in this study is based on a Linkam BCS196 cryobiology stage (Linkam, Guildford, UK), attached to an upright fluorescence microscope, following the approach

published by van Driel et al [22]. The microscope is equipped with a multi-channel filter set allowing blue, green and red wavelengths to be imaged in a single optical configuration. This allows quick multi-channel acquisitions without changing the filter cube for each channel, thereby limiting potential image shifts between colour channels due to different optical alignments.

We have modified the existing cryo-stage aiming to minimize contamination of the sample due to moisture condensation, and to maintain a low sample temperature while using an objective with a shorter WD (Fig. 1A-D). The stage is mounted to the microscope body using a custom-made stage adapter plate (Fig. 1A-C). The microscope objective is inserted into the cryo-stage through a specially designed lid. The lid consists of an aluminium cooling chamber and a teflon tube with a screwed inset that encloses the objective (Fig. 1B,C). Various insets with different inner diameter allow the use of different dry objectives. The aluminium cooling chamber can be filled with dry ice, thereby maintaining the stage surfaces and lid at an operating temperature of -60°C . The use of dry-ice rather than LN eliminates potential vibrations due to boiling. Due to the low operating temperature at the stage surface it was necessary to replace all lubricants for the mechanical parts of the Linkam system by aircraft grease certified for very low temperature use. This permits smooth movement of the sample within the cold stage. The imaging chamber is flushed with dry, cold nitrogen gas from a dewar equipped with an electric heating device that evaporates LN (See Fig. 1D for a schematic of the cooling system). This generates a slight overpressure that prevents humidity from entering the stage through any opening. The objective cools down during operation resulting in a measured temperature of around -80°C at the front lens. To prevent condensation and frost build-up, which would otherwise interfere with the imaging, we flush the back lens of the objective with the dry gas exhaust from the Linkam pump (Fig. 1D). The objective withstands the repeated cooling without any observed effect. Rapid, forced warming however is not advisable as the introduced material expansion and tensions may damage the optics.

The sample itself, mounted on a standard size 3.05 mm diameter EM grid, is held in place by a specially designed brass grid holder (Fig. 2A), which fits into the Linkam sample carrier. The grid cannot be kept in position by a large clip, since this would get in the way of a short WD objective closely approaching the sample. Instead the grid is held in place by a brass weight covering the rims of the grid with only a very thin layer of material. This allows us to make use of a $50\times$, 0.95 NA, $300\text{ }\mu\text{m}$ WD objective. Using the weight to hold the grid in position does not appear to damage the vitrified ice layer. A central, 2 mm diameter hole in the grid holder allows bright field illumination to focus on the sample. The size of this hole also determines the practically usable field of view for cryo-FM: imaging areas of the grid not located above this hole is difficult due to fluorescence background from the brass holder.

Once inserted into the stage, the base of the grid holder sits directly on a cooled silver block to maintain it at the required temperature. The Linkam stage includes a temperature sensor within the silver block and regulates the flow of LN to maintain a stable temperature during imaging. Grids were imaged by cryo-FM for up to 80 minutes but typically between 10 and 20 minutes. No decline in FM image quality was apparent during imaging, making it

unlikely that condensation is collecting within the light path. Grids imaged by cryo-FM for up to 80 minutes and then by cryo-EM did not show noticeably more ice contamination than grids which were imaged only by cryo-EM, suggesting that ice contamination during FM imaging is not a limiting factor during normal use of the stage.

We made minor modifications to the loading station, used to transfer the sample into the cryo-stage [22], to facilitate grid handling and minimize contamination during the transfer procedure. Compared to the original setup, we reduced the size of the LN bath, changed the orientation of the cartridge base to simplify the loading procedure and added a block to hold grid boxes (Fig. 2B). The loading box is able to slide on a table adjacent to the microscope body (Fig. 1A) to directly attach to the door of the cryo-stage for loading.

2.2 Workflow for cryo-FM/EM of vitrified samples

The following protocol describes the cryo-FM/EM workflow for plunge-frozen samples, which is schematically illustrated in Figure 3.

EM grids must be selected which have a support film that emits as little auto-fluorescence as possible. They should also have grid-wide landmarks visible in both FM and EM to facilitate the approximate orientation of the images between the two imaging systems. Appropriate landmarks are those found on commercial “finder-grids” or a random pattern of grid squares in which the support film has been punctured with an eyelash. Fiducial markers that can be easily and unambiguously identified in the EM, and that fluoresce in multiple wavelengths, are pre-adhered to the support film of the grids. Multi-coloured fiducials can be distinguished from the signal of interest because they are visible in all colour channels, but importantly, they also fluoresce in the same channel as the signal of interest. All correlations can thus be calculated using a single colour channel, minimizing inaccuracies due to chromatic aberrations, or due to drift during multi-channel acquisition.

The sample is mixed with fiducial gold, applied to the grid, blotted and vitrified by plunge freezing according to standard protocols. Fiducial gold is required in the sample to ensure accurate coordinate transformation between different EM magnifications.

The grids are transferred under LN to the cryo-FM loading-station and one grid is placed into the grid holder. The carrier is then inserted into the cryo-stage. The grid is inspected in bright field imaging mode to obtain an overview of the grid and its ice conditions and to find the centre mark, and its orientation. A stack of fluorescence images is then acquired by manually translating to between 20 and 50 grid squares, and adjusting the focus at each position before collecting multi-colour channel images. The spatial location of the grid landmarks, the relative position of the most promising grid squares and the corresponding best images in the recorded stack, are all noted down after FM imaging.

The grids are then transferred to the electron microscope. An initial overview montage of the entire grid is collected, identifying the grid-wide landmarks and defining the relative orientation of the map relative to the FM images. For each grid-square of interest, montage maps are recorded at an intermediate magnification. Ideally, this is the minimum magnification at which fiducial beads and gold fiducials can be clearly identified, thereby

maximizing in each image the number of fiducial markers that can be used to calculate the coordinate transforms.

Within an individual grid square, prominent features visible in both FM and EM images, such as empty holes in the carbon or individual fiducials or clusters of fiducials, are used to generate a coarse alignment of the two images. These features are marked as “registration points” within the SerialEM software that is used to control the electron microscope [26]. The “Transform Points” command in SerialEM’s Navigator panel can then be used to apply a general linear transformation to the set of marked coordinate points to register the two images [27]. The software thereby transforms the FM image coordinates to the coordinate system it uses to control the electron microscope stage. Fluorescent spots of interest can thus be directly selected in the imported fluorescence images and the SerialEM software will automatically acquire high-magnification images or tomograms at those spots. This approach was also applied by Briegel et al [18].

After data collection is completed, the transformations that precisely relate the coordinates of features in the acquired FM and EM images are calculated from the respective pairwise coordinates of fiducial markers in the FM images and in the intermediate magnification EM maps, according to the procedure described in [16] (Fig. 4). Using this transform, the x and y coordinates of the signal of interest in the EM maps are determined (Fig. 4A,B). The coordinates of the signal of interest in the intermediate-magnification EM map are then transformed to the high-magnification EM image or tomogram based on the positions of gold fiducials in both images using an analogous procedure [16].

2.3 Precise localization of fluorescent bacteriophages in a vitrified sample

Holey carbon copper grids (C-Flat 2/2, 300 mesh, Protochips, Raleigh, NC, USA) were pre-loaded with 100 nm Tetraspeck fluorescent microspheres (Life Technologies, Carlsbad, CA, USA) as fluorescent fiducials. The grids were incubated for 2 min on 13 μ l droplets of Tetraspecks, diluted 1:10 in PBS, after which the grids were washed by briefly placing them on three droplets of water. On moving between droplets the grid was not blotted, instead the excess liquid was removed by physically shaking. Landmarks were generated by punching random grid squares using an eyelash. Grids were glow-discharged, and a solution of p22 bacteriophage particles, containing genetically incorporated GFP attached to the scaffold protein [28,29], was mixed with 10 nm colloidal gold particles on the grid, blotted, and vitrified by plunge-freezing. Each bacteriophage particle is estimated to contain 100-330 copies of the scaffold protein, and on average 280 copies of GFP [28,29].

FM images were collected in blue, green and red channels at an effective magnification of 62.5 $\times$, giving a pixel size of 139 nm and a field of view of 340 μ m. EM was carried out on a Titan Krios (FEI Company, Hillsboro, OR, USA) transmission electron microscope operating at 120 kV, and equipped with a 4k Ultrascan (Gatan, Inc., Pleasanton, CA, USA) CCD camera. To collect montage images of the grid we used the SerialEM software [26]. Initial overview montages of the entire grid were taken at a magnification of 100 $\times$ (pixel size 342.8 nm). Maps of the individual grid squares of interest were collected as 6 $\times$ 6 montages with a 50% spatial overlap at 2300 $\times$ magnification (pixel size 4.86 nm). FM images of each grid square were extracted from the FM dataset as 800 $\times$ 800 pixel sub-images from the

channel of interest. These images were imported into SerialEM and aligned to the EM image based on prominent landmarks as described above. Fluorescent signals likely to correspond to individual phage particles were then selected in the FM image for automatic acquisition of high magnification EM images at each of those positions (magnification 22,500 $\times$, pixel size 3.78 Å).

We acquired a dataset of a total of 261 high-magnification images based on fluorescent spots. In almost all cases we could identify a bacteriophage particle in the field of view of the acquired micrograph. Out of this dataset we selected 115 spots for high-accuracy correlation based on ice quality and whether the bacteriophage was sufficiently far away from nearby fiducial beads to exclude crosstalk in the fluorescence images.

The coordinates of the fluorescent spots corresponding to phage particles were derived from the FM image with sub-pixel precision by 2D Gaussian fitting of the spots using a self-written MATLAB (MathWorks, Natick, MA, USA) routine. The signals were sufficiently bright to be easily detected: the mean intensity of the fluorescent signals was 3000 counts above background, while the background noise in the holes in the carbon fluctuated with a standard deviation of 80 counts. The coordinates of the fluorescent spots were then transferred into the intermediate EM map, as described previously [16] and in the previous section, and then further transferred to the high magnification EM image with the help of the gold fiducial markers. Based on these calculations, the software superimposes a circle that indicates the expected position of the bacteriophage particle on each of the high-magnification EM images covering a spot of interest (Fig. 4C).

2.4 Measuring the accuracy of the correlation procedure

We estimated the accuracy of the coordinate transformation by treating an individual fiducial bead as the feature of interest, excluding it from the set of fiducial marker pairs and using the remaining ones to calculate the transform that relates the FM and EM images. We then measured the accuracy of the correlation by comparing the predicted position of the excluded bead to the true location of the bead in the EM image (Fig. 5). This “leave-one-out” cross-validation test of the fiducial markers’ coordinates is identical to the procedure we previously described for correlation of resin-embedded samples [16]. A plot of the predicted position of the beads, relative to the true position (the origin) is shown in Fig. 5A. The deviation of the predicted position of the excluded bead from the true position can be treated as two independent experiments for each coordinate axis. We found a normal distribution for the spatial deviation of the two coordinate axes, centered on the origin, with standard deviations of 56 and 67 nm in x and y. This placed 80% of correlations within 87 nm of their predicted positions and 50% of correlations within 49 nm of their predicted positions (Fig. 5B).

The accuracy can also be tested by measuring the deviation of the predicted position from the center of the bacteriophage particle, easily recognizable in the high magnification cryo-EM images. The predicted positions of the bacteriophages, relative to their true positions (the origin) are shown in Fig. 5A. Overall 80% of correlations were within 67 nm of their predicted positions and 50% of correlations were within 41 nm of their predicted positions (Fig. 5B). The square root of the variance of the deviation from the origin is 34 and 43 nm in

x and y, respectively. While this distribution also showed normal behaviour, the distribution is centred on a position approximately 20 nm away from the origin. This indicates that there is also a small systematic offset in the prediction of the centre of the bacteriophage within the fluorescence image that contributes to the overall error.

3 Technical Details

3.1 Production of p22 particles

GFP-containing p22 bacteriophage particles were expressed as described in [28]. Cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.6, 100 mM NaCl, 2 mM MgSO₄), lysed by freeze-thawing and sonication. Cell debris was removed by centrifugation at 4,000× g for 20 minutes and the supernatant was pelleted through a 20% sucrose cushion for 2 h at 125,000× g. The pellet was resuspended in 1 ml 50 mM Tris pH 7.6, 25 mM NaCl, 2 mM EDTA, dialyzed into the same buffer to eliminate any residual sucrose and treated with 0.1 mg/ml DNase (final conc.). The sample was loaded onto a 5-20% sucrose gradient, centrifuged at 180,000× g for 35 minutes and the bacteriophage-containing fraction was dialyzed against the same buffer overnight.

3.2 Fluorescence microscope

The BX51 WI (Olympus, Hamburg, Germany) fixed-stage microscope is equipped with an Andor neo sCMOS camera (Andor Technology, Belfast, UK) operated using the iQ software (Andor Technology, Belfast, UK). An MPLAPO 50×/0.95 (Olympus, Hamburg, Germany) objective was used, with an additional 1.25× magnification lens between microscope and camera. A solid-state emitter CoolLed (CoolLED Ltd., Andover, UK) operating at 380 nm and 470 nm with a common 387/478/555 excitation filter (all filters: Semrock, Rochester, NY, USA) was used for illumination. For excitation of RFP or Cherry a metal halide lamp HXP120 (Leistungselektronik JENA, Jena, Germany) with a 580/23 excitation filter was used. Red, green and blue colour channels were imaged using the same filter cube with a triple dichroic mirror 405/488/594 and a triple emission filter 425/527/685. This set of filters was chosen to provide optimal performance for GFP while allowing fast acquisition of other channels without changes in the beam path. These filter bands and illumination are sub-optimal in the red channel, but are sufficient for detection of brighter red signals such as those in the multi-colour beads.

4 Discussion

We have described a cryo-FM setup that can employ short WD objectives with a high NA up to 0.95. The angular aperture of the objective, the sine of which is represented by the NA, contributes with its square to the capability of the objective to collect photons, and the NA contributes linearly to resolution in FM imaging. Consequently, a 0.95 NA objective compared to a 0.75 NA objective, has approximately 2.2 times increased sensitivity and a resolution that is improved by a factor of 1.27. In the cryo-stage, contamination due to ambient humidity is minimal and devitrification risks are minimized: we have imaged grids for 80 minutes without detecting deterioration of the sample quality. This cryo-stage is therefore appropriate for applications that demand high-sensitivity and high-resolution.

The bacteriophages that we have imaged contain an estimated 280 copies of GFP (though this varies between particles) [28,29]. They therefore give a bright signal. We found that the mean peak intensity of the bacteriophage signal was 3000 counts, which is approximately 34 times higher than the fluctuations in the background. While we have not quantified the detection limits of the cryo-FM setup, these observations suggest that a cluster of 30 GFP molecules would give a signal with an intensity multiple standard deviations above background under these imaging conditions. We note that these imaging conditions have not been fully optimized for sensitivity.

When carrying out fiducial-based correlations of resin-embedded samples, the accuracy of the method is limited by local sample deformations that are not properly accounted for during the transformation of coordinates. When carrying out correlations for vitrified samples, there are fewer local distortions, and the optical performance of the microscope becomes the limiting factor for the correlation accuracy. Lateral optical distortions such as spherical aberrations will be taken into account by the transform relating the FM image to the EM image. The effect of any chromatic aberrations (which may be exacerbated by low temperature), are largely eliminated by measuring the positions of the object of interest and the fiducial markers within the same FM image (in the same colour channel). For a typical plastic embedded sample, the fiducial beads deviate from their predicted positions with standard deviations of between 90 and 100 nm (measured from the dataset described in [30]), compared to 65 nm for the cryo-FM/EM set-up. The correlative procedure is therefore more accurate for the vitrified sample than for the resin-embedded sample.

The cryo-FM/EM correlation accuracy as assessed using the bacteriophage particles is better than the accuracy determined using the fiducial beads (Fig. 5). A number of factors may combine to cause this difference. These include firstly that the bacteriophage position is calculated using one more fiducial bead than the position of the “leave-one-out” fiducial bead. Secondly, some of the beads are positioned at the edge of the bead cloud (the set of beads used to calculate the correlation) where their position may be predicted less reliably than the bacteriophage, which is typically located towards the centre of the bead cloud. Thirdly the fluorescence signal from the bacteriophage is approximately 2.5 times brighter than that of the fiducial beads, allowing its centroid position to be more accurately determined. Fourthly, the background fluorescent signal fluctuates slightly more on the carbon film, where the fiducials are located, than in the holes in the ice, where the bacteriophages are located.

The accuracy with which we can localize the bacteriophage is high (80% were within 67 nm of the true position, and 50% within 41 nm): it is significantly below the size of the pixel in the fluorescence microscope (139 nm). When we looked at the distribution of all predicted positions we found it to be centred at a position approximately 20 nm away from the true position (Fig. 5A). This small error originates from an uncertainty in the measurement of the position of the centroid of the fluorescent spot from the bacteriophage, relative to the positions of the fiducial beads. This indicates that part of the error in localizing the bacteriophage is systematic. The reasons for this offset are unclear. One possible source is the difference in the spectral properties of the fluorophores on the beads and the GFP in the virus particles, combined with a change in the chromatic correction properties of the

objective at low temperatures. Another potential source of error is the difference in z-height, within the sample, between the position of the beads and that of the bacteriophage. Combined with an asymmetry in the point-spread function of the objective due to low-temperature this could account for the systematic error in correlation. Further research will be required to identify the source of this error. These observations highlight the challenges in carrying out very high-accuracy correlative procedures. Nevertheless, for larger viruses with sizes of over 100 nm, it would be possible to determine the source of a fluorescent signal with sub-viral precision.

The application of high-NA cryo-FM to vitrified samples will allow correlated FM/EM to be carried out where high-sensitivity and high-resolution is required. In combination with a fiducial bead-based correlation procedure, fluorescent signals can be localized with very high accuracy within the EM image. Further characterization of the behaviour of fluorescent molecules and high-performance optics for FM at low-temperature is required, but we envisage that there will be applications of cryo-FM even when not correlated with cryo-EM. In particular, cryo-FM has potential to be adapted to allow the use of super-resolution FM techniques, which will likely benefit from photophysical effects at low temperatures that include reduced bleaching. Current limitations of current cryo-FM systems including our setup clearly lie in mechanical stability and the optical performance at the low temperatures required to preserve the sample. Further improvements in this area will enhance both image quality and correlation accuracy. Work being carried out in multiple labs and industry will result in optimized cryo-stages in coming years and thus facilitate application of correlative cryo-FM/EM methods to a variety of biological problems.

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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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Fig. 1. The cryo-FM setup.

A Overview photograph of the cryo-FM setup. The Linkam cryo-stage is mounted onto an upright microscope. On top of the stage is the modified lid with its aluminium cooling chamber (c1) and the Teflon tube (c2) that surrounds the objective. The dewars containing LN (d1, d2) and the pump (p) are connected to the cryo-stage by tubes (t1-t4). The table on the right (t) allows the transfer box (Fig. 2) to slide and connect to the stage door (d). **B** Higher-magnification view of the cryo-stage (st) and lid. Cooling line t1 supplies LN from dewar d1 (not shown, see Fig. 1A) to the silver-block that supports the sample. The LN

evaporates within the tube and leaves the chamber through t2 to the pump (Fig. 1A). This gas is vented via t4 to the back lens of the objective back to prevent condensation. Line t3 purges the chamber with dry gas. The adaptor plate connecting the stage to the microscope (ap), and the door to the stage (d) are marked.

C Illustration of the cryo-FM system in cross-section. The grid sits in the grid holder that in turn sits on a silver block inside the stage chamber. The grid holder is moved with a sample carrier that is inserted through the door (d) on the side. The actual location of the EM grid (sample) is indicated in magenta.

D Schematic depiction of cooling and purging flows in the cryo-stage. The dark blue colour indicates LN, light blue indicates dry nitrogen gas flows. The silver block that supports the sample is cooled by LN, that is transported through tubes t1 and t2 by a pump (p) and evaporates while in the tubes. The chamber is purged with cold nitrogen gas from a dewar (d2) with an incorporated electric heater. The resulting overpressure prevents humidity from entering. The objective back lens is flushed with dry gas (t4) to prevent condensation. The stage surfaces are cooled by dry ice in the cooling chamber of the lid (c1). The transfer box (Fig. 2B) is filled with LN and can be tightly attached to the stage. The sample carrier, while present during imaging, is not illustrated to improve clarity.

Fig. 2. The grid holder and transfer box.

A A schematic representation of the two-part holder for standard EM grids. The grid sits on the lower brass disc and is held in place by the weight of the upper brass disc whose thin cover allows imaging with a small working distance objective. The off-centre holes in the upper disc are to facilitate manipulation with tweezers. The grid holder fits into the Linkam sample carrier.

B A photograph showing the transfer box, attached to the microscope, from above. The sample carrier sits in its loading station inside the transfer box. The brass well is filled with

LN while in operation. The carrier is loaded into the cryo-stage via the door (d) and slides into the chamber. The door can be closed before the transfer box is removed for imaging. A brass block (b) holds the grid box during loading.

Fig. 3. The cryo-FM/EM work-flow and time schedule for plunge-frozen specimens.

The central column lists the individual steps in the workflow, with their estimated duration indicated in the left column. A continuous line indicates a single procedure, whereas a dashed line indicates positions where the procedure can be paused. Grey boxes indicate the key sessions of the experiment.

Fig. 4. The high-accuracy correlation procedure

A Fluorescence image showing signals from multi-colour fluorescent beads and from green fluorescent bacteriophages within one EM grid square. Merge of red, green and blue channels. Towards the edges of the grid square, close to the copper bars of the grid, the thick ice and subsequent high concentration of fluorescent particles leads to a high background signal.

B Magnified view of the region indicated in A. The signals from individual multi-colour fluorescent beads (Tetraspecks) that are used as fiducial marks are indicated by yellow circles. The signal from a feature of interest is indicated by a red square.

C Intermediate-magnification cryo-EM map of the region shown in B. The field of view of panel B is marked by the dashed black lines. Corresponding fiducial beads are marked as in B (yellow circles). The red square is centred on the predicted coordinates of the feature of

interest. Dark regions on the regular holey carbon support film (black arrows) are remnants of sucrose from particle purification. Red circles mark crystalline ice particles.

D Magnified regions of C showing four typical fiducial beads (left panels) and four ice particles (right panels) as seen in the intermediate-magnification EM maps. The homogenous size and shape and distinct density of the fiducial markers allows them to be distinguished from ice particles. White arrows indicate 10 nm gold fiducial beads used to align the intermediate magnification maps to the high-magnification micrographs. Width of image windows: 400 nm.

E High magnification cryo-EM image centred on the predicted position of the feature of interest, showing a p22 bacteriophage particle. The indicated circle revealing the predicted coordinates has a radius of 50 nm. White arrows indicate 10 nm gold particles.

Fig. 5. Measurement of the accuracy of the correlation procedure.

A The predicted position of all beads (blue), and all bacteriophages (green), are marked relative to the true position of the bead or bacteriophage. The true position is the origin. For comparison, the outline of a bacteriophage is shown to scale (red hexagon), as is the outline of a single 139 nm pixel in the fluorescence image (red square).

B The localization error is plotted against the percentage of correlations with errors smaller than that localization error. Blue curve: the deviation of the fiducial bead position in EM

from their predicted position applying a leave-one-out cross-validation. Green curve: the deviation of the predicted position of the bacteriophage particles from their true position.