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Correlative Cryo-electron Tomography and Optical Microscopy of Cells

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Abstract

The biological processes occurring in a cell are complex and dynamic, and to achieve a comprehensive understanding of the molecular mechanisms underlying these processes, both temporal and spatial information is required. While cryo-electron tomography (cryoET) provides three-dimensional (3D) still pictures of near-native state cells and organelles at molecular resolution, fluorescence light microscopy (fLM) offers movies of dynamic cellular processes in living cells. Combining and integrating these two commonly used imaging modalities (termed correlative microscopy) provides a powerful means to not only expand the imaging scale and resolution but also to complement the dynamic information available from optical microscopy with the molecular-level, 3D ultrastructure detail provided by cryoET. As such, a correlative approach performed on a given specimen can provide high resolution snapshots of dynamic cellular events. In this article, I review recent advances in correlative light microscopy and cryoET and discuss major findings made available by applying this method.

Introduction

Both fluorescence light microscopy and electron microscopy have been instrumental for understanding the structure and function of biological processes and have provided a wealth of information regarding protein localization and dynamics and the cellular ultrastructure. Cryo-electron tomography (cryoET) has emerged as a powerful imaging technique that allows three-dimensional (3D) visualization of the molecular landscape within cells, at 2–4 nm resolution, in a close-to physiological state [1]. By applying this method, significant structural detail has been obtained for bacterial cells [2, 3], cellular compartments [4], and purified or abundantly budding virus particles [5–7]. With recent developments in subvolume averaging from cellular tomograms, the structures of native protein assemblies, at resolutions below 2 nm, are now possible [8, 9]. However, several issues associated with cryoET have limited its potential in advancing cellular structural biology. First, the inherent low contrast of unstained frozen-hydrated specimen in cryoET, combined with specimen radiation sensitivity, makes it difficult to locate areas of interest inside a cell before cryoET analysis. Second, cryoET delivers only still pictures of cells. Third, although providing molecular resolution, cellular tomograms offer a very limited field of view, typically only ~10% of a cell's area and restricted to very thin regions, typically those < 300nm thick.

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Thus, the vast majority of a eukaryotic cell is not accessible to cryoET alone. Finally, due to the crowded cellular environment, in the absence of EM labels, individual proteins and macromolecular complexes are not readily distinguishable at the current resolution, with the exception of a few, very large complexes [10, 11].

On the other hand, fluorescence light microscopy (fLM) offers a complementary set of imaging capabilities. It provides time-resolved, large scale (whole cell) visualization of the dynamics of individual fluorescent protein(s) of interest in living cells. Thus, fluorescence imaging has been employed to investigate the temporal and spatial distribution of specific molecular players in a variety of cellular processes. Recent advances in super-resolution fluorescence microscopy imaging, which apply switchable probes, as in PALM and STORM [12, 13], or elegant illumination strategies, as in STED and SIM [14–16], have further improved the resolution of fLM by an order of magnitude and have enabled a better understanding of many cellular processes [17, 18]. Yet, the limited spatial resolution of fLM often prevents definitive localization of proteins relative to the cellular ultrastructure or organelles. Further, only a few molecules can be visualized simultaneously with fLM, in contrast to the full repertoire of macromolecules that can be visualized with cryoET.

Therefore, a correlative approach that combines the strength of these two complimentary imaging modalities is highly desirable. In such a way, specific features highlighted by fluorescent signals are identified and located by fLM and then used to guide the acquisition of high resolution 3D structural data by cryoET, which in turn complement the dynamic information obtained by live-cell fLM. Using correlative microscopy, one can not only visualize the dynamics of the cellular process in a whole living cell but can also zoom in to observe the molecular details of the cellular context of the process to gain novel insights into the process.

Correlative light and electron microscopy (CLEM) has become a powerful approach for structural and functional analysis of cellular processes in a single experiment. CLEM was achieved primarily using either chemically or cryo-fixed, resin embedded and sectioned specimen at room temperature, typically after immunolabeling [19, 20] or photoconversion of diaminobenzadine into an electron-dense marker, such as miniSOG [21•]. Abundant application of this approach has yielded many significant findings and has provided insights into some fundamental biological processes and their underlying molecular mechanisms [19, 22•]. Here, I focus on CLEM studies performed at cryogenic temperature (cryo-CLEM), which is more challenging, but allows fluorescent signals to be examined in frozen-hydrated cells preserved at near-native condition for subsequent cryoET analysis [23]. In this review, I outline the workflow for cryo-CLEM, highlight recent technical advances and the major findings obtained using this approach, and discuss the future prospects for using correlative microscopy in conjunction with superresolution (see article by Moerner), cryoFIB milling (see article by Baumeister), and/or genetically expressed EM markers, to advance our imaging capability for investigating cell signaling and cellular processes.

Cryo-CLEM implementation

Cryo-correlative light and electron microscopy (i.e. cryo-CLEM) is a multistep experiment involving protein fluorescent-labeling, live-cell confocal microscopy, sample vitrification, cryo-fluorescent light microscopy (cryo-fLM), and cryoET data collection and analysis. A typical workflow for cryo-CLEM is illustrated in Figure 1 (steps 1 through 6). Cells grown directly on indexed, gold EM grids (Fig. 1a) are typically labeled with one or more fluorescent dyes and examined with time lapse, live-cell confocal imaging (Fig. 1b) to assess the dynamic behavior of the labeled protein(s) of interest. At a specific dynamic stage of interest, the cells are preserved at near-native condition by rapid vitrification (Fig. 1c). Cryo-

fLM is performed to identify the area of interest using a cryo-light microscopy (cryo-LM) stage that maintains the sample in the frozen-hydrated state (Fig. 1d). Specific features highlighted by fluorescent labeling are identified and located, and their coordinates are transferred to the electron microscope for acquisition of high resolution 3D structural data using cryoET (Fig. 1e). The final stage of cryo-CLEM (Fig. 1f) involves structural and functional analysis of the biological process of interest by integrating the 3D structure data with dynamics information obtained from the live-cell imaging (step 6). In many cases, when the structures of interest are stable, the fLM images recorded in live cultures (Fig. 1b) can be used directly to guide cryoEM localization of the area of interest (step 2). However, for highly dynamic features, a cryo-LM stage, to retain the vitrified state, is needed for direct correlation.

Recent technical advances in cryo-CLEM

Cryo-CLEM holds great promise as a tool for structural and functional analysis of cellular processes under close-to-native conditions. But, it is technically challenging, particularly during the cryo-fLM imaging stage of the method. Although a number of cryo-LM stages have been developed and successfully applied [24–30], the approaches carried out with these stages have involved separate LM and EM instruments and, thus, required specimen transfer, making the specimen vulnerable to physical damage and contamination. To minimize this risk during grid transfer, various techniques have been applied, one of which involved the development and application of an integrated specimen cartridge holder that allows both cryo-fLM and cryoEM to be performed within a single specimen holder, eliminating the need for grid transfer [25•, 28]. Very recently, however, an integrated cryo-CLEM sample stage (ILEM²) became available to address this issue. Specifically, the ILEM², combines cryo-fLM and cryoEM into a single instrument using a retractable optical element and a rotating specimen holder, to circumvent the need for specimen transfer [31•]. This “2 in 1” approach greatly simplifies sample handling and navigation between the two modalities, resulting in faster throughput and greater efficiency. The ILEM concept was first demonstrated with room temperature correlation [32] and has recently been commercialized (FEI’s Tecnai™ with iCorr™; FEI Company, USA). However, to image dynamic events, the two-step correlation approach, as illustrated in Fig. 1(b–d), is still required.

Along with these instrumentation and grid transfer issues, correlating fLM images and EM images is a non-trivial task, due to the vastly different image intensities, contrast, magnification, and resolution. Because correlation is commonly done manually, with the aid of finder grids, the accuracy of the correlation is susceptible to error. For this reason, along with the ILEM², Faas and colleagues developed an integrated software, which applies a coordinate-based, pre-calibrated relocation system [30], making navigation between the two imaging modalities much easier [31•]. The alignment can be further simplified and improved by including fiducial markers that provide contrast for both fLM and cryoEM [33, 34].

Correlating structure, function and dynamics of cellular processes

As an emerging technique, correlative cryoET and fLM has been successfully applied to investigate cell adhesion [35•], mitosis [36], neuronal synapses [30], and the cytoplasmic microtubule network [27]. In these cases, correlation experiments were primarily carried out to facilitate cryoET data acquisition; the structure of interest was localized using cryo-fLM to allow meaningful reconstruction of cellular macromolecular structures. More recent efforts, as exemplified in the studies described below, have focused on using cryo-CLEM to connect the cellular ultrastructure to the function and/or dynamics of a biological process,

providing new insights into how molecular composition and dynamics are related to cellular structure and function.

Cryo-CLEM provides a revised view of a prokaryotic structure

A large body of fLM studies employing fluorescent protein fusion tags had shown that the bacterial actin homolog, MreB, polymerizes into long helical filaments that encircle diverse rod-shaped bacteria. These studies led to a predominate view in the field that a helical cytoskeleton plays a central role not only in cell shape determination but also in chromosome segregation, cell polarity, motility, and growth [37]. Jensen's group recently applied cryo-CLEM to understand the function and structural organization of MreB filaments in a number of rod-shaped bacteria [38•]. Cells overexpressing GFP-MreB were labeled with the membrane dye FM4-64, plunge frozen on an EM finder-grid, and imaged by cryo-fLM, then by cryoET. Through this correlative approach, they confirmed that MreB forms large bundles of filaments in the cytoplasm (Fig. 2a). However, in contrast to the vast number of previous fLM observations, no MreB helical filaments were seen encircling the cells. Instead, the extended helical patterns consistently seen in the previous fLM studies were observed to be artifacts due to overexpression of the MreB fusion protein (Fig. 2b) [39]. More recent live-cell optical microscopy studies also indicate that MreB does not form extended helices but rather forms discrete patches that move circumferentially around the cell [40–43]. Taken together, these results shed new light on the structure and function of MreB, which is now understood to be a dynamic entity that moves around the cell envelope as part of the cell wall synthetic machinery, instead of a large, rigid helical scaffold.

Cryo-CLEM to understand viral dynamics in cells

Application of cryo-CLEM to dynamic processes, such as viral entry, egress, and budding during infection, in cells arrested during a particular functional state of interest, can provide high-resolution still pictures of specific stages of dynamic processes. For example, Ibricic and colleagues applied live-cell imaging along with cryoET to understand the dynamics and structure of herpes simplex virus 1 (HSV1) in neurons during egress. Specifically, they first determined the peak for anterograde virus transport by live-cell imaging, then used cryoET structural analysis to determine the structure of viruses during this peak [44••] (Fig. 3). Their analysis revealed that HSV1 capsids were transported independently of the viral envelope and that the secondary capsid envelopment occurs in axon terminals. Further, using cellular cryoET combined with subvolume averaging [8, 45], they were able to visualize the tegument density that is exclusively associated with the capsid vertices on intra-axonal capsids and to determine that both DNA-packed capsids and empty capsids undergo active axonal transport. Yet, due to technical difficulties, the live-cell imaging and cryoET were not performed on the same specimen, thus the structure and function correlation was indirect.

A direct correlation between the time-lapse, live-cell imaging and high resolution cryoET at the level of individual viral particles was first accomplished by Jun et al. during visualization of single native HIV-1 cores within cells at an early stage of infection [25••]. Studying early HIV-1 infection events in the context of the dynamical interplay between virus and host cell components is particularly challenging [46], due to the small size of the viral particle (diffraction limited), the rarity of infection, and the highly dynamic nature of infection events. Catching a few single infecting viral particles (100 nm) in a vast host cell (50 μ m) at the right moment for cryoET analysis would be nearly impossible without correlative live-cell imaging. Using high-speed confocal live-cell light microscopy followed by cryo-fLM and cryoET on the exact same viral particle (see Fig. 1 for workflow), Jun et al., for the first time, directly detected and imaged individual HIV-1 particles at different infection stages by high resolution cryoET [25••]. While a majority of stationary HIV-1

particles retained viral envelope and were either attached to the cell surface or internalized in vesicular compartments, fast moving particles appeared to be released into the cytoplasm after membrane fusion and to be en route to the nucleus (Fig. 4). Thus, with correlative imaging, Jun et al. were able to connect viral dynamics in a live cell to the structure of viral components in the cellular context during infection. The group further obtained direct evidence to suggest that a hyperstable HIV-1 capsid mutation, E45A, delayed capsid uncoating compared to the wild-type capsid. Together, these results demonstrate the importance of correlating 3D live-cell imaging with cryoET when rare and dynamic cellular processes are to be analyzed or when cryoET data will be more meaningful with information about the dynamics or a functional state of cell under investigation.

Concluding remarks

Correlative cryoET and optical microscopy is an emerging powerful approach to visualize dynamic cellular processes. Recent technical advances in cryoEM and cryoET, including direct electron detectors and phase plates, provide a positive prospect on achieving multiscale and high resolution in analyzing biological systems in the coming years. However, cryoET, like other TEM techniques, suffers from a basic limitation: sample thickness, which needs to be <300 nm. Given this limitation, cryoET and correlative imaging have been more successfully applied to small prokaryotes and viruses. Many eukaryotic cells are too thick to be imaged by cryoET, and currently, only the peripheral regions of the cell are suitable for 3D structural analysis by cryoET. Reducing the thickness of frozen-hydrated specimens, to allow access to the vast interior of the cell, by vitreous sectioning [47–49] or by cryo-focused ion beam (FIB) milling [50–52] (see review article by Baumeister) would greatly expand the capability of cryoET and correlative imaging of eukaryotic cells.

CryoET offers the potential to visualize the full repertoire of macromolecular complexes in a cellular landscape at 2–4 nm resolution. However, discerning these complexes in a crowded cellular environment [53], possibly with a template-matching approach [54], is a challenge. Nonetheless, with the advancements in super-resolution optical imaging, identification of supramolecular complexes (10–20 nm) in correlated cellular tomograms may become possible [22•]. Dual labels which are both fluorescent and electron dense are highly desirable to achieve correlation at the single molecule level. Future development in this area, in particular genetically encoded GFP-equivalent analogues for cellular cryoET, such as tetracysteine-ReAsh [55] and metallothionein [56], will greatly enhance the ability to dissect the molecular mechanisms of cellular events.

TEM with a liquid cell technology, which potentially allows unconventional live-cell imaging by TEM [57], combined with fLM, could offer new possibilities for time-resolved high resolution views of cellular processes, including viral infection, cell signaling, and transcription/translation processes. It is also expected that integrating additional imaging modules, such as atomic force microscopy [58], x-ray tomography [59, 60], and scanning electron microscopy [61], combined with molecular and cell biology methods, will provide complementary spatial, temporal, structural, biochemical and biophysical details that allow a thorough and comprehensive understanding of cellular and molecular phenomena.

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Highlights

Describe a workflow for correlative fluorescent microscopy and cryo-electron tomography

Highlight technical advances in cryo-correlative microscopy (cryo-CLEM)

Correlate structure, function and dynamics of cellular processes with cryo-CLEM

Figure 1.

An illustrative workflow of correlative light microscopy and cryo-electron tomography of cells. The steps include: (a) culturing of eukaryotic cells on carbon-coated gold EM index grids in a glass-bottom petri dish; (b) time-lapse, confocal live-cell imaging of a dynamic cellular process, such as viral infection, directly performed on cells grown on EM grids; (c) arresting the cellular process at a particular state by vitrification (plunge-freezing); (d) localizing the target of interest using fluorescence signals by cryo-fLM for subsequent cryoET; (e) cryoET data acquisition and 3D reconstruction of the identified target; and (f) 3D structural analysis and interpretation, as well as correlating the high resolution cellular structure with the dynamics of the fluorescently labeled proteins (step 6). For stable and static structures, live cell fluorescence images can also be used to guide cryoEM localization (step 2), without cryo-fLM.

Figure 2.

Visualizing bacterial cytoplasmic MreB filaments. (a) Correlated cryo-fLM (inset) and cryoET of a *V. cholerae* cell overexpressing GFP-MreB. Red is the membrane-dye FM4-64 and green is GFP-MreB (inset) with the dashed lines (red and green) representing the thresholded boundaries of each signal, respectively. Cryo-electron tomogram, acquired from the same cell, display a large filament bundle running through the cytoplasm (arrows), which corresponds to the GFP-MreB signal. Scale bars, 1 μm in the fLM inset and 200 nm in the cryoET slice [38•]. (b) MreB helices are an artifact of the YFP tag when overexpressed in *E. coli* cells. No filaments were seen near the membranes of wild-type cells (WT) or cells overexpressing native MreB (MreB OE), but many helical and other filaments were found just inside the membranes of cells overexpressing YFP-MreB (YFP-MreB OE, YFP-MreB hyperOE). Inset, 3D model of a YFP-MreB OE cell's inner membrane (blue) and YFP-MreB filaments [39].

Figure 3.

Transport of HSV1 capsids in neurons during egress. (a) Phase contrast image of a hippocampal neuron grown on a holey carbon support film for 7 days. A neurite (white arrow) and cell body (asterisk) are indicated. (b) Time-lapse fluorescence images of a mid-axon region of a HSV1(KOS)-GFPVP26 infected neuron at 16 hours postinfection, recorded at the indicated time (seconds). The left image shows an overlay of the fluorescence signal with the bright field image. Arrows indicate the positions of individual viral particles. (c) CryoEM projection image of an intact axon at 16 hours post-infection, showing cytosolic C- and A-capsids (boxed with solid and dashed lines, respectively). (d–f) Slices through the reconstructed tomographic volume obtained from the area of interest in (c). pm: plasma membrane; mt: mitochondria; black arrows: microtubules. Scale bars: 6 μm in (a), 5 μm in (b), and 200 nm in (c–f) [44••].

Figure 4.

Correlation between HIV-1 dynamics and 3D structure of host cell complexes during HIV infection by combining confocal live cell microscopy, cryo-fLM, and cryoET. (a–c) Direct visualization of a “trafficking” native HIV-1 core within the infected cell. The HIV-1 particle was tracked in 3D for 40 min (yellow trace) with time-lapse live cell confocal imaging (a), followed by plunge-freezing and cryo-fLM imaging to localize the particle, and subsequent low-dose cryoEM imaging of the correlated target region (marked with “*”) at low magnification (b) and cryoET (c). A 4 nm thick tomographic slice from the 3D tomographic volume acquired from the correlated cellular region (* in b) shows an intact intracellular HIV-1 core (boxed) situated next to a vesicle and in the vicinity of cellular filaments and microtubules. Scale bars: 10 μm in (a) and (b), 100 nm in (c). (d–f) Direct visualization of a “stationary” native HIV-1 virion (circled in d) bound to a HeLa cell by live cell imaging with 3D tracking (d, red trace, stationary, blue trace, dynamic, 30 min), low (e) and high (f) magnification cryoEM imaging of the correlated area (boxed in e). The enveloped, static HIV-1 particle was observed bound to a cell protrusion (arrow). Scale bars, 5 μm in (d) and (e), and 100 nm in (f) [25••].