Recent Developments in Correlative Super-Resolution Fluorescence Microscopy and Electron Microscopy

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INTRODUCTION

The recently developed correlative super-resolution fluorescence microscopy (SRM) and electron microscopy (EM) is a hybrid technique that simultaneously obtains the spatial locations of specific molecules with SRM and the context of the cellular ultrastructure by EM. Although the combination of SRM and EM remains challenging owing to the incompatibility of samples prepared for these techniques, the increasing research attention on these methods has led to drastic improvements in their performances and resulted in wide applications. Here, we review the development of correlative SRM and EM (sCLEM) with a focus on the correlation of EM with different SRM techniques. We discuss the limitations of the integration of these two microscopy techniques and how these challenges can be addressed to improve the quality of correlative images. Finally, we address possible future improvements and advances in the continued development and wide application of sCLEM approaches.

Keywords: correlative light and electron microscopy, correlative super-resolution fluorescence and electron microscopy, electron microscopy, super-resolution fluorescence microscopy

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physical or chemical properties (Go et al., 2021).

Although SRM techniques increase the spatial resolution by an order of magnitude or even more than the diffraction-limited resolution, they still cannot resolve many cellular structures with a size of a few nanometers. Although the recently introduced MINFLUX (minimal photon fluxes) nanoscopy, which has been developed based on a synergistic combination of the specific strengths of STORM/PALM and STED, has demonstrated spatial resolutions in the range of 1-3 nm for structures, application of such SRM remains limited to mapping the labeled fluorophores, not the real target itself (Gwosch et al., 2020; Schmidt et al., 2021). Such small-sized ultrastructures in cells are often observed by electron microscopy (EM) because transmission and scanning electron microscopy (TEM and SEM, respectively) allow higher resolutions than SRM. These methods also allow subcellular organelles, including lipid membranes, to be imaged at a nanometer resolution without specific labeling, implying that EM can provide spatial information of “reference space.” However, molecule-specific imaging is limited by EM, and only a small number of specific molecular structures with characteristic shapes can be identified in EM images. Examples of such structures are the mitochondria, endoplasmic reticulum, and microtubules. In addition, live-cell imaging with EM remains challenging owing to the requirement of an ultra-high vacuum and conductive metal coating during imaging (Kim et al., 2021b).

Because of the complementary advantages and disadvantages of SRM and EM, they have recently been combined to simultaneously obtain the spatial locations of specific molecules with SRM and the context of the cellular ultrastructure revealed by EM, such that we can benefit from the advantages of both techniques. This integration of SRM and EM techniques is often referred to as correlative SRM and EM (sCLEM) (Hauser et al., 2017; Kim et al., 2015). Although the combination of SRM and EM remains challenging owing to the incompatibility of their sample preparations, sCLEM can add new dimensions to SRM techniques by allowing meaningful correlations with ultrastructural details (Hauser et al., 2017; Kim et al., 2015). To obtain multidimensional information from the same target, different types of SRM have been combined with various EM methods. These have been utilized for imaging various cellular targets over the past decade. The sample condition determines the choice of the EM type to be combined with the SRM. For example, for thin samples or for samples for which the surface is of interest, metal replica TEM or SEM imaging of whole-mount samples would work well (Kim et al., 2015). Meanwhile, if the samples are embedded in resins and sectioned using an ultramicrotome, the interior regions of these samples can be imaged by TEM or BSE-SEM (backscattered electron-SEM). To preserve their native ultrastructures, the samples can be cryofixed and cryo-sectioned, instead of being chemically fixed or resin embedded (Ahn et al., 2020).

In this review, we discuss the technological advances in sCLEM depending on their types (STORM, PALM, STED, and SSIM) and their applications to various biological samples. In sCLEM, it is challenging to obtain optimal quality for both SRM and EM because the samples prepared for both imaging methods are not compatible. For example, the strong fixation conditions typically required for ultrastructure preservation in EM sample preparation can result in fluorescence quenching. This results in a degradation in the SRM image quality. However, mild fixation conditions cannot be used to minimize the photon loss of fluorophores, hence ultimately compromising the quality of both SRM and EM. We describe the limitations of these techniques and attempts to overcome these challenges. Lastly, we discuss possible future improvements and advances in sCLEM.

### Correlative Super-Resolution Fluorescence Microscopy and Electron Microscopy

#### Correlative STORM and EM

STORM employs the stochastic activation of individual fluorescent molecules at different times, allowing spatially resolved single-molecule imaging (Fig. 1A). Given that the fluorescence intensity from a single molecule is the most important factor in determining the resolution of STORM (Kim et al., 2021a), it is important to minimize the photon loss of fluorophores resulting from the strong fixation during EM sample preparation for correlative STORM and EM imaging.

To overcome this limitation, many researchers have performed correlative STORM and EM imaging by separating the fluorescence-incompatible conditions for EM sample preparation from STORM imaging (Chung et al., 2021) (Fig. 1B, Table 1). For instance, Löschberger et al. (2014) first obtained the STORM image of nuclear pore complexes, and then fixed the samples with 2.5% glutaraldehyde (GA) and stained them with 2% osmium tetroxide (OsO₄) for subsequent SEM imaging. Kim et al. (2015) also separated the treatment of fluorescence-quenchable fixation and staining reagents from STORM imaging to obtain high-quality STORM images of virus-infected cells by completely avoiding photon loss in STORM imaging (Fig. 1C). Similarly, Sulaiman et al. (2013) reported good quality STORM images for the kidney glomerular capillary loop by fixing samples with 2% GA and depositing platinum to form a replica for EM imaging. By completely separating the fluorescence-incompatible conditions for EM sample preparation, such as strong fixation and metal staining steps from STORM imaging, neither STORM nor EM image quality was compromised although those imaging regions were limited to the surface structures of the samples. Additionally, Wojcik et al. (2015) developed the correlative STORM and SEM method using graphene coating to bypass fluorescence-quenchable EM preparation steps. Graphene-coated wet cell samples could be imaged by both STORM and SEM because graphene is transparent to both light and electrons. Owing to the electrically conductive and liquid-impermeable properties of the graphene layer, the graphene-coated wet cell samples did not need dehydration or coating with a conductive layer for EM imaging. Hence, fluorescence quenching has been avoided. In these methods, most of the sample preparation protocols for STORM and EM were based on standard protocols used for each imaging modality; hence, neither STORM nor EM images were substantially compromised. However, these approaches are limited to imaging structures that are relatively close to the cell surface.
To image the interior regions of thick biological samples, they need to be embedded in resins and thin-sectioned. However, it is challenging to obtain good quality for both STORM and EM imaging with such thin-sectioned samples because an embedding procedure, including strong EM-related fixation, can affect both the fluorescence in STORM images and the ultrastructure preservation in EM images. To minimize these effects, researchers have optimized sample preparation for STORM and EM (Fig. 1D). For example, Perkovic et al. (2014) chose the SiR fluorophore from a screening test of synthetic fluorophores because of its ideal photoswitching properties under a specific buffer, pH, and laser intensities. They also used cryo-fixation without the use of fluorescence-quenchable OsO₄ for resin-embedded section samples. Although they could improve the fluorescence intensity in STORM images under the optimized conditions, the subsequently processed EM image quality substantially degraded owing to insufficient EM fixation. Kim et al. (2015) also developed correlative STORM and EM imaging methods for resin-embedded sectioned samples (Fig. 1E). They tested various types of resins, polymerization strategies, EM fixation additives, and stains to find the ideal conditions to minimize fluorescence quenching and preserve the ultrastructure. They also noticed inhibition of the photoswitching property when the dyes were embedded in resins due to the blocked access of thiol to dye molecules. Because a fast photoswitching rate of the dye molecules is required for super-resolution imaging, they exploited a chemical etching approach to expose the epoxy-embedded dye to the imaging buffer, thereby optimizing photoswitching and improving the quality of super-resolution fluorescence images (Fig. 1E).

**Correlative PALM and EM**

PALM is a single-molecule localization microscopy technique similar to STORM, and these two techniques are essentially similar in terms of single-molecule localization based method (Jung et al., 2020) (Fig. 2A). Although these techniques currently use both fluorescent organic dyes and fluorescent proteins (FP), we mainly discuss the PALM process that uses FP to focus on its different properties from organic dyes under EM sample preparation. The first correlation of PALM and EM was reported when PALM was first developed, wherein Betzig et al. (2006) showed the application of correlative PALM and TEM for imaging the matrix of mitochondria using
cryo-section samples (Table 2). The use of cryo-sections allows the FP to preserve their fluorescence intensities, even in the section (Fig. 2B). Kopek et al. (2012; 2013) also used the cryo-section strategy to correlate PALM and SEM (Fig. 2C). Likewise, most correlative PALM and EM imaging has been conducted using cryo-sections, as FP in the cryo-sections remain in the hydrated state at an optimal pH, thus avoiding its destruction and fluorescence quenching. However, the methods that use cryo-sections suffer from a low electron contrast, resulting in low-quality EM images.

To better preserve the ultrastructure of biological samples, correlative cryo-PALM and cryo-EM imaging methods have also been attempted with the recent development of the cryo-imaging technique. Because an elevated temperature at any step can induce the growth of ice crystals, and hence, structural distortion, cryo-PALM imaging after cryofixation allows the samples to remain in their native states in both the PALM and EM images. Although several researchers could obtain high-quality EM images with ultrastructure preservation using this method (Chang et al., 2014; Hoffman et al., 2020; Liu et al., 2015), their PALM images suffered from low labeling density due to the low-resolution effect of cryo-imaging. Although recent studies have made remarkable headway in achieving good quality cryo-PALM images, further improvements in the resolution of cryo-PALM imaging are anticipated to be better correlated with higher resolution EM images (Hoffman et al., 2020).

Another method to preserve the ultrastructure during correlative PALM and EM imaging is the use of resin embedding, which is typically used for the EM imaging of sectioned biological samples (Fig. 2D). Watanabe et al. (2011) developed correlative PALM and BSE-SEM methods based on a conventional resin embedding method. To minimize the fluorescence quenching and destruction of FP, they used a very low concentration of OsO4 and a hydrophilic acrylic resin. Compared to organic dyes, FP can be easily perturbed by EM fixatives because of the acidic and oxidizing fixation reagents, such as OsO4; therefore, it is challenging to preserve the fluorescence from FP in the resin-embedded sections. Although Johnson et al. (2015) reported that the fluorescence from mGFP, mVenus, and mRuby2 can be better preserved by the addition of tannic acid, the quality of the PALM image remains poor in the correlative images (Fig. 2E).

To overcome this limitation, various engineered variants of EosFP that fluoresce and photoconvert normally under heavily fixed conditions have been reported. For example, Paez-Segala et al. (2015) developed fixation-resistant mEos4a and mEos4b by reducing the surface side-chain reactivity and improving the thermodynamic stability. They demonstrated that these fixation-resistant Eos variants are
compatible with 0.5%-1% OsO4-fixed plasticized sections and enable super-resolution imaging. Subsequent work by Fu et al. (2020) also reported another OsO4-resistant mEos that survived Epon embedding after OsO4 treatment and reported the improved quality of correlative PALM and EM images for mitochondria and nuclear lamina.

Another method to avoid the fluorescence loss of FP during sample preparations for EM and to preserve the high EM contrast, is to conduct all EM-related sample preparations after PALM imaging, as discussed above. For instance, Van Engelenburg et al. (2014) combined iPALM (interferometric PALM) with SEM to image ESCRT subunits at HIV assembly sites. In addition, Sochacki et al. (2014) employed metal-replica electron tomography for obtaining 3D correlative images with iPALM to completely separate the EM-related sample preparations from those for the iPALM images. Although these methods can avoid the loss of photons or photoactivation properties of FP, the EM images are limited to the cell membrane surface owing to the use of metal replicas and SEM (secondary electron-SEM).

Correlative STED/SSIM and EM

Both STED and SSIM can be categorized as SRM techniques that use patterned illumination: STED uses negative patterning, whereas SSIM uses positive patterning (Fig. 3A). STED microscopy employs the stimulated emission at the area surrounding the focal spot of the excitation laser by the donut-shaped depletion light, allowing a sharpened focal spot of the image. SSIM improves the image resolution up to ~50 nm by the reconstruction of multiple snapshots taken by scanning and rotating the positive patterning of the excitation light on the sample. Because these techniques do not require the photoswitching or photoactivation of fluorophores such as in STORM or PALM, respectively, the quality of STED and SSIM images in the EM correlative images can be less sensitive to the EM-related sample preparation than in those methods (Table 3). For example, Watanabe et al. (2011) reported a better quality of STED images, with higher labeling density than that for the PALM images for mitochondria in their correlation images with BSE-SEM.

There have been substantial efforts to preserve both the fluorescence and ultrastructure of samples when correlating EM with STED or SSIM because they play important roles in determining the quality of SRM and EM images. For instance, de Waal et al. (2018) separated the SSIM imaging step from the EM-related sample preparation steps to image lipopolysaccharide and its association with fibrin fibers, which is similar to the previously discussed correlative STORM/PALM and EM methods. Al Jord et al. (2014) also performed EM-related sample preparation, such as EM-fixation, heavy metal staining, resin embedding, and sectioning, after 3D SIM imaging. To align these two different images for the unsectioned and sectioned sample, they employed grided coverslips for a reasonable 3D alignment at a high precision. To obtain a high fluorescence
intensity from fluorophores, Müller et al. (2017) proposed a protocol for correlative SIM and TEM using self-labeling proteins such as SNAP- and CLIP-tag and organic dyes instead of FP (Figs. 3B and 3C). They demonstrated this protocol for the imaging of age-defined granule morphology and degradation with high fluorescence contrast. Wurm et al. (2019) employed post-embedding immunolabeling rather than pre-embedding immunolabeling to avoid the destructive effects of permeabilization and fluorescence quenching during the embedding process. This method led to a high signal-to-noise ratio of the fluorescence signal; however, the changes in antigenicity resulted in a low labeling density in STED images.

Several dual-contrast reagents have also been developed to better correlate STED/SIM with EM images. For instance, Prabhakar et al. (2018) applied fluorescent nanodiamonds (FNDs) to correlative STED and TEM as dual-contrast fiducial markers because they are known to be EM-detectable electron-dense materials and are compatible with STED imaging (Fig. 3D). Although FNDs allow a resolution of 6 nm during STED imaging, they are limited in their use just as fiducial markers because of the lack of target specificity. As a dual-contrast marker with target specificity, Tian et al. (2020) designed an iridium (III) complex, clr-Tub, which can specifically bind to $\alpha\beta$-tubulin active pockets. They demonstrated the capability of this marker for correlative STED and TEM, but the STED images displayed a limited labeling density for microtubules. As dual-contrast markers are beneficial for providing a precise correlation between SRM and EM images, further developments of such markers to obtain a high labeling density and high binding specificity are necessary for

| Table 2. Comparison of reported studies on correlative PALM and EM |
|-----------------|-------------|-------------|-------------|-------------|---------------|-------------|
| EM              | Sample type in SRM | Sample type in EM | Fluorophore | Target | Remarks | Reference |
| SEM             | Cryo-sectioned | Au/Pd coated | mEos2 | Mitochondria in 3T3sw cells | Gold nanospheres deposited and ITO sputtered coverslips | Kopek et al., 2013 |
| Unembedded      | Carbon and Au/PD coated | PSCFP2, Alexa 647 | COS-7-HIV Gag-FLAG | Gold nanorods deposited and SiO$_2$ sputtered coverslips | Van Engelenburg et al., 2014 |
| BSE-SEM         | Carbon coated | Dendra, tdEos | C. elegans worms | High-pressure freezing | Watanabe et al., 2011 |
| GMA resin embedded | Au/Pd coated | mEos4a, mEos4b | Lamin A in 3T3 cells | High pressure freezing | Paez-Segala et al., 2015 |
| FIB-SEM         | Methylcellulose embedded | psCFP2, Alexa 647 | Membrane in PC12-GR5 cells | Gold nanorods deposited and SiO$_2$-sputtered coverslips | Sochacki et al., 2014 |
| TEM             | Cryo-sectioned | Methylocellulose embedded | dEosFP, GFP | Mitochondria in 3T3sw cells | Stained with 3% uranyl acetate before EM imaging | Betzig et al., 2006 |
|                 | Metal replica, critical-point dried | Membrane in PC12-GR5 cells | Sin1-Dendra2$^+$ | Red-shifted FluoSpheres shows the best performance for correlative PALM and TEM | Fathima et al., 2021 |
| HM20 resin embedded | HM20 resin embedded | GFP, mVenus, mRuby2 | HEK293T cells | High pressure freezing | Johnson et al., 2015 |
|                 | E6 Vero cells expressing nsp3-GFP | GFP | High pressure freezing | Tuijtel et al., 2017 |
| Epon resin embedded | Epon resin embedded | mEosEM | Mitochondria in CHO cells | Gold nanoparticles used as markers | Fu et al., 2020 |
| GMA resin embedded | GMA resin embedded | mEos4a, mEos4b | Lamin A in 3T3 cells | High pressure freezing | Paez-Segala et al., 2015 |
| Cryo-EM         | Cryo-sectioned | Dronpa | Mitochondria in HEK293 cells | High pressure freezing | Liu et al., 2015 |
| Cryo-ET         | Plunge frozen | Plunge frozen | PA-GFP | Bacteria (Myxococcus xanthus) | PA-GFP photoactivatable at 80K | Chang et al., 2014 |
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high-quality correlative images.

PERSPECTIVES ON FUTURE sCLEM IMAGING TECHNIQUES

Considerable attempts have been made to develop sCLEM techniques. This new and fast-growing field of research could be applied in a wide range of areas as it may provide valuable information that has not been obtained through conventional microscopy. In particular, this method has been effectively applied to visualize specific locations of protein coats or viruses on the cell membrane ultrastructure with high mo-
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molecular specificity because the advantages of both SRM and EM images can be obtained without compromised image quality (Bykov et al., 2016; Kim et al., 2015; Sochacki and Taraska, 2021). In addition, the application of this method to nanomedicine is promising. Andrian et al. (2021) recently reported the capability of correlative SRM and TEM to image the functional moieties of nanoparticles at the single-particle level, which is crucial for nanoparticle activity. The technique can be further employed for nonbiological materials, which are generally more robust than biological samples (Hauser et al., 2017). For example, surface-plasmon-enhanced catalytic activity has been visualized by sCLEM at the nanoscale within single plasmonic nanostructures (Zou et al., 2018). By spatially localizing catalytic products using SRM and correlating this with high-resolution nanostructure geometry using EM, nanoscale catalytic properties can be quantified.

Although recent studies have reported promising results using sCLEM as discussed, further improvements are needed to enhance the correlative image quality. First, the precise alignment between SRM and EM images remains challenging owing to the dissimilarity of contrast, spatial resolution, and sample volume owing to shrinkage. Because this constraint arises from the different contrast reagents used for each imaging technique, the use of a dual-contrast reagent could allow improved alignment between the two images with high precision. Although several studies have recently reported the development of dual-contrast correlative imaging agents, including fluorophore-labeled gold nanoparticles, quantum beads, spectrally shifted dark-red FluoSpheres (Fathima et al., 2021), silica-coated gold nanoparticles (Fokkema et al., 2018), uranyl acetate (Tuijtel et al., 2017), FNDs (Prabhakar et al., 2018), and iridium (III) complex ([Ir(phen)2(dppz)]2+), (Chiral Os(II) Polypyridyl Complexes) (Tian et al., 2020), the development of dual-contrast markers with high labeling density, small size, and high binding specificity for diverse targets remains a future challenge. For example, Huang et al. (2020) developed cell-impermeable chiral Os(II) polypyridyl complexes as dual-contrast nuclear DNA imaging reagents. Although they successfully demonstrated the correlational imaging of nuclear DNA with this agent in diffraction-limited LM images, they failed to demonstrate the applicability

### Table 3. Comparison of reported studies on correlative STED/SIM and EM

| SRM   | EM  | Sample type in SRM | Sample type in EM | Fluorophore | Target | Remarks | Reference       |
|-------|-----|--------------------|-------------------|-------------|--------|---------|----------------|
| STED  | SEM | Lowicryl resin embedded | Lowicryl resin embedded | Alexa 594, Atto 590, Abberior STAR RED | Alpha-tubulin and variant surface glycoprotein (VSG), in T. brucei cells | High pressure freezing, Ultrathin sectioning | Wurm et al., 2019 |
|       | TEM | LR White and GMA resin embedded | Carbon coated | Citrine | Caenorhabditis elegans worms | High-pressure freezing | Watanabe et al., 2011 |
|       | TEM | Unembedded | Epon resin embedded | MitoTracker Green | Mitochondria | Fluorescent nanodiamonds (FNDs) used as dual contrast marker | Prabhakar et al., 2018 |
| EF-TEM| SEM | Epoxy embedded | Carbon coated | clr-Tub (cyclometalated Iridium (III) complex) Alexa 488 | Microtubules in HepG2 cells Lipopolysaccharide in whole blood | Ultrathin sectioning 1% OsO4 fixation after STORM imaging | Tian et al., 2020 |
|       | SEM | Unembedded | Carbon coated | mEmerald-ER3 JF525, Alexa 647 | Lipopolysaccharide in whole blood | High-pressure freezing | de Waal et al., 2018 |
| FIB-SEM| Cryo-sectioned | Eponate 12 resin embedded, reembedded in durcupan | mEmerald-ER3 JF525, Alexa 647 | ER, mitochondria, transferrin in COS-7 cells | High pressure freezing | Hoffman et al., 2020 |
| TEM   | Cryo-sectioned or epon resin embedded | Methylcellulose embedded or epon resin embedded | SNP | Insulin secretory granules | High pressure freezing | Müller et al., 2017 |
|       | Unembedded | Epon resin embedded | Cen2–GFP | Ependymal progenitors | Relocation of grid-imprinted glass | Al Jord et al., 2014 |
|       | Araldite resin embedded | Os-1/TeCP ([Os(phen)2(dppz)]2+, Chiral Os(II) Polypyridyl Complexes) | Cell nuclear DNA | Mesh copper grids and fluorescent beads used for correlation | Huang et al., 2020 |
of this method for correlative SIM and TEM imaging, probably owing to insufficiently optimized sample preparation for correlation. Because the SRM image quality in the correlative image considerably depends on the fluorescence intensity, as discussed, studies need to optimize sample preparation for better fluorescence preservation when using dual-contrast reagents for sCLEM imaging. Next, it is necessary to overcome the current limitations of sCLEM imaging at a cryo-state as a promising future direction for sCLEM imaging (Jun et al., 2019). Although recent works have demonstrated the feasibility of cryo-SRM imaging and the correlation with cryo-EM, there is still a large resolution gap between cryo-SRM and cryo-EM owing to the limited resolution of SRM images at a cryo-state (Wolff et al., 2016). Such a low resolution of cryo-SRM results from the use of a long-working distance, with nonimmersion objective lenses at a cryo-state. Thus, it is necessary to develop an objective lens that can drastically increase the photon yield in cryo-SRM imaging. In addition, the altered photoswitching or photoactivation properties at a cryo-temperature can limit SRM imaging (Wolff et al., 2016). Therefore, a better understanding of the properties of fluorophores at cryo-temperatures is also needed to optimize the SRM imaging conditions at a cryo-state. The development of SRM at a cryo-state could drive the correlation between cryo-SRM and cryo-electron tomography (cryo-ET). This could enable the 3D visualization of biomolecules and organelles at molecular resolution under close-to-physiological conditions. Although improving the stability of the sample stage and optical aspects in the cryo-imaging setups remain challenging, it is anticipated that structural-functional analyses can be performed in the most innate form of the samples at the nanoscale level (Jun et al., 2019).

Collectively, with the advances in sCLEM, we foresee extensive opportunities for its application to clarify demanding questions in a wide range of areas by providing new and multidimensional information about targets.

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AUTHOR CONTRIBUTIONS
D.K. supervised the overall process and wrote the manuscript, and D.J. designed the figures and the table. All authors discussed the related studies, drafted and contributed to the final manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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